Differences in callosal and subcortical volumes and associated neurobehavioural deficits in children with prenatal alcohol exposure

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Declaration

I, Stevie Crystal Biffen, hereby declare that the work on which this thesis is my own unaided work both in concept and execution, and that apart from the normal guidance from my supervisors, I have received no assistance.

This thesis has been presented by me for examination for the degree of Doctor of Philosophy in Neuroscience.

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Abstract

Certain high-risk communities in the Western Cape Province of South Africa where heavy maternal prenatal alcohol consumption is perpetuated by historical and societal challenges, have some of the highest prevalence rates of fetal alcohol syndrome (FAS) in the world. FAS has lifelong behavioural and cognitive consequences. Neuroimaging research aims to link deficits in brain structure and function to behavioural outcomes. Manual tracing is considered the gold standard of neuroanatomical volumetric analysis. Combined with neurobehavioural testing it can provide links between structure and function, but is time consuming and labour intensive. Automated segmentation programmes, such as FreeSurfer, are a faster alternative. The challenge is creating automated programmes that can provide results that are comparable to manual tracing, especially in a clinical sample. The aims of this thesis were to investigate (1) the effects of prenatal alcohol exposure (PAE) on the sizes of the caudate nucleus, nucleus accumbens, hippocampus and corpus callosum (CC) and potential relations of regional volumes with IQ and verbal learning, (2) to compare the performance of manual and automated segmentation methods in identifying alcohol-related changes in brain morphometry, and (3) to examine the effects of PAE on inter-hemispheric transfer during adolescence and potential relations of CC size with inter-hemispheric transfer deficits.

Participants for this project were recruited from the Cape Town Longitudinal Cohort for whom alcohol exposure data were gathered prospectively from the mothers during pregnancy using the timeline follow-back approach. Participants had been diagnosed previously by two expert dysmorphologists as either control, non-syndromal heavily exposed (HE), partial FAS (PFAS) or FAS. High-resolution T1-weighted images were acquired using a sequence optimized for morphometric neuroanatomical analysis on a Siemens 3T Allegra MRI scanner for 71 right-handed children (9 FAS, 19 PFAS, 24 HE and 19 non-exposed controls) from this cohort at ages 9-11 years. Bilateral caudate nuclei, nucleus accumbens and hippocampi and the CC were manually traced using Multitracer. FreeSurfer was used for automated segmentation. All structures were segmented with both FreeSurfer versions 5.1 and 6.0 to compare progress within development of automated segmentation algorithms. Associations of volumes from manual tracing with IQ and performance on the California Verbal Learning Test-Children's Version (CVLT-C) were also examined. Inter-hemispheric transfer was assessed using a finger localization task (FLT) administered to 74 participants (12 FAS, 16

PFAS, 14 HE, and 32 controls) from the same cohort at ages 16-17 years. Of these, 34 participants had completed MRI at 9-11 years.

Higher levels of PAE were associated with reductions in CC area, as well as bilateral volume reductions in caudate nuclei and hippocampi, effects that remained significant after controlling for alcohol-related reductions in TIV (total intracranial volume). Amongst dysmorphic children (FAS/PFAS), poorer performance on the CVLT-C was related to larger hippocampi and smaller CC. Smaller CC was also associated with lower IQ and partially mediated the effect of PAE on IQ. Manual and automated comparisons showed good agreement in the caudate nuclei, which are simpler to segment, moderate to good agreement in the smaller, more complex nucleus accumbens and hippocampi, and poor agreement in the CC. The latter is not surprising, however, in view of the fact that manual tracing measured the average area of the CC on a mid-sagittal slice, while FreeSurfer measures CC volume over a number of contiguous slices. After controlling for confounders and adjustment for smaller TIV, the latest FreeSurfer version 6.0 provided evidence of alcohol-related volumetric brain reductions comparable to manual segmentation. Only the most severely affected children with FAS demonstrated inter-hemispheric transfer deficits, with the number of transfer-related errors tending to increase with decreasing CC volume among children with PAE.

This study confirms and extends evidence of PAE-related decreases in subcortical and CC size and that callosal volume partially mediates alcohol-related impairment in IQ. Although FreeSurfer v 6.0 achieves automated segmentations that are comparable to manual tracing, even in a paediatric clinical sample, performance is more reliable in some structures than others. Improvement and standardization of CC segmentation is especially important given the vulnerability of the CC and its critical role in domains affected by PAE, including verbal learning, IQ and inter-hemispheric transfer of information.

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"Happiness can be found, even in the darkest of times, if one only remembers to turn on the light."

- Albus Dumbledore, Harry Potter and the Prisoner of Azkaban-

Thank you to everyone who helped me find happiness throughout this thesis and in my journey up to this point. My family (Nigel, Machelle and Sookie Biffen, Mitchell Eva, Travor and Jean Vincent and all the rest of the crazy clan) and my friends (you know who you are, you have my tear stains all over your stuff and my laughter in your hearts) have been like Eärendil, the brightest of stars, and find unique and wonderful ways to light my way through the darkest of times. You are my favourite heroes and I love and thank you all.

List of Abbreviations

AA	Absolute alcohol (oz)
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Caudate	Caudate nucleus
CC	Corpus callosum
CUBIC	Cape Universities Brain Imaging Centre
CUD	Crossed-uncrossed differences
CVLT-C	California Verbal Learning Test-Children's Version
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
FAS/PFAS	The group comprising children with either a diagnosis of FAS or PFAS
FLT	Finger localization task
GM	Grey matter
HE	Heavily exposed non-syndromal participants
HIV	Human immunodeficiency virus
IQ	Intelligence quotient
CUD	Crossed-uncrossed differences
MRI	Magnetic resonance imaging
HIV	Human immunodeficiency virus
MEMPRAGE	Multi-echo magnetization prepared rapid gradient echo
MRI	Magnetic resonance imaging
NA	Nucleus accumbens
PAE	Prenatal alcohol exposure
PFAS	Partial fetal alcohol syndrome
ROI	Region of interest
SES	Socioeconomic status
TIV	Total intracranial volume
WISC-IV	Wechsler Intelligence Scale for Children-Fourth Edition
WM	White matter

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Preface

This dissertation uses neuroimaging and cognitive testing to investigate associations of prenatal alcohol exposure (PAE) with subcortical and corpus callosal structure and function.

Chapter 1 presents the motivation, rationale and background of the overall study. It introduces fetal alcohol spectrum disorders (FASD) and its prevalence in the Western Cape Province of South Africa. In this chapter, I also briefly summarize the current literature on the effects of PAE on the selected regions of interest investigated in this dissertation.

Chapter 1 is followed by three individual manuscripts comprising chapters 2 to 4. Chapter 2 presents findings on the effects of PAE on select subcortical structure volumes and corpus callosum (CC) area in 9-11-year-old children with FASD, and associations of PAE-related changes in size with IQ and verbal learning. In chapter 3, a comparison is presented of manual tracing and automated segmentation in measuring the volumes of the bilateral caudate nuclei, nucleus accumbens and hippocampi, as well as the area of the CC. The effects of PAE on inter-hemispheric transfer of tactile information in adolescence are examined in chapter 4, as well as the potential role of PAE-related changes seen in CC size in pre-adolescence on inter-hemispheric transfer performance. The assimilation of these three papers into one body of work does result in necessary repetition, as each article follows the format of an introduction, methods, results and discussion section.

The study described in chapter 2 has been published in the journal “Frontiers in Neuroanatomy”. Manual tracing of structural MRI was used to measure the volumes of bilateral caudate nuclei, nucleus accumbens and hippocampi, and the area of the CC. Our interest in these neuroanatomical structures was based on previous findings showing inconsistent results within the selected subcortical structures and that white matter (such as the corpus callosum) is especially vulnerable to PAE. This paper also examines how structural changes may relate to performance on IQ and CVLT-C scores. This article was co-authored by Christopher Warton, Nadine M. Lindinger, Catherine E. Lewis, Steven R. Randall, Christopher D. Molteno, Joseph L. Jacobson, Sandra W. Jacobson and Ernesta M. Meintjes.

Professor Ernesta Meintjes contributed on overall project supervision, collaboration on the study design, oversight of data acquisition and analysis, and collaboration on interpretation and write-up of the study. Professors Sandra and Joseph Jacobson's contribution to the paper included designing the study, supervising recruitment, maternal interviews and child assessments, as well as collaboration on data analysis, interpretation of the findings and write-up of the paper. Doctor Christopher Warton contributed to student supervision, devised the manual tracing protocol, provided consultation on neuroanatomical findings and trained me, along with co-author Steven Randall, to conduct manual tracing. Professor Christopher Molteno collaborated on study design, and administered maternal interviews, which included sociodemographic information and alcohol, smoking and drug ascertainment. Doctors Nadine Lindinger and Catherine Lewis performed the neuropsychological assessments.

Chapter 3 is the second manuscript and investigates the validity of automated FreeSurfer subcortical and corpus callosum segmentation in pre-adolescent children in studies of FASD and is currently being reviewed by co-authors, Christopher Warton, Christopher D. Molteno, Joseph L. Jacobson, Sandra W. Jacobson and Ernesta M. Meintjes. It has not been submitted for publication as of yet.

Chapter 4 investigates the effects of PAE on CC area and inter-hemispheric transfer of tactile information using a finger localization task (FLT). This manuscript is currently under review by co-authors Christopher Warton, Christopher D. Molteno, Joseph L. Jacobson, Sandra W. Jacobson and Ernesta M. Meintjes.

The final chapter discusses the overall findings from the study as a whole. The structural differences seen in the aforementioned regions of interest and how these relate to functional correlates found in this study are explored further. Similarities and differences of the current findings and the existing literature are discussed with an emphasis on linking structure and function. The findings of both manual and automated segmentation are discussed in reference to how different tracing methods may impact clinical findings, using the current data as an example. Finally, the limitations of the study are reviewed and conclusions are drawn.

Chapter 1: Introduction

Maternal drinking during pregnancy has lifelong consequences for their offspring (Jones and Smith, 1973) due to resulting brain damage that can range from microcellular, neurochemical and cellular dysfunction to gross structural abnormalities (Ikonomidou, 2000; Olney, 2004; Spadoni et al., 2007; Lebel et al., 2011; Donald et al., 2015). As such, cognitive and behavioural dysfunction also varies in terms of degree of developmental delay or disability, and severity and range of learning disabilities (Astley et al., 2009b; Mattson et al., 1998). Due to the varying effects of prenatal alcohol exposure (PAE), the term fetal alcohol spectrum disorders (FASD) has been coined as an umbrella term under which the entire range of PAE-related outcomes fall. Therefore, FASD itself is not a medical diagnosis, but rather refers to the spectrum of disorders that may result from PAE, including fetal alcohol syndrome (FAS), partial FAS (PFAS) and alcohol-related neurodevelopmental disorders (ARND). Of these, Fetal Alcohol Syndrome (FAS), which is characterized by growth deficits, small head circumference, distinctive craniofacial dysmorphology (small palpebral fissures, flattened philtrum and thin vermillion) and behavioural and cognitive problems (Jones and Smith, 1973; Hoyme et al., 2005), is the most severe.

The effects of PAE are seen in both developed and developing countries, albeit at different prevalence rates (May et al., 2000; May and Gossage, 2001). May and colleagues (2009) estimated that, in the western world and the United States children are affected by FAS at a rate estimated to be between 0.05 (May and Gossage, 2001) to 7 (May et al., 2009) per 1000 live births. In contrast, certain high-risk communities in the Western Cape Province of South Africa have been found to have an FAS prevalence rating that is 18 to 141 times higher than this, with a rate of 39.2 to 42.9 per 1000 children aged 6 to 7 years old (May et al., 2000). Another study found an FAS prevalence rating of 65.2 to 74.2 per 1000 children in the first grade (Viljoen et al., 2005). The authors stated that the prevalence of FAS in this community had increased within 2 to 3 years and this made it one of the highest recorded prevalence rates of FAS in any community in the world. It is important to study the effects of FASD on the developing brain in these communities most heavily impacted by the condition where the patterns of drinking, socioeconomic conditions and demographics are vastly different to that of the more widely studied communities in the developed world.

Prevalence studies have found that, within the Western Cape Province of South Africa, rates are highest amongst the Coloured population (May et al., 2005, 2000; Viljoen et al., 2005). This population is of mixed Asian, African and European ancestry (May et al., 2005, 2000; Viljoen et al., 2005). These authors have asserted that their findings could be extrapolated to other communities in South Africa and that the high prevalence found in the Western Cape population is attributable to the use of the "Dop" system (May et al., 2005, 2000; Viljoen et al., 2005). The word "dop" comes from the informal Afrikaans word for alcoholic beverage and refers to the practice where for centuries the farm employees involved in the growth of grapes and production of wine were partially paid with wine, which was used as a primary form of entertainment in the form of binge drinking (May et al., 2005, 2000; Viljoen et al., 2005). Although now illegal, the effects of this heavy alcohol use are still prevalent in the population (Burden et al., 2005; May et al., 2005, 2000; Viljoen et al., 2005). Studies have shown that mothers of children diagnosed with FAS are still more likely to live in rural environments, work on farms and be of lower socioeconomic status (May et al., 2005, 2000; Viljoen et al., 2005). Even as urbanisation occurs, the social consequences of this historical practice persist.

FASD has devastating, lifelong effects on brain function, as evidenced by numerous studies demonstrating deficits in a broad range of cognitive and behavioural assessments, including those measuring IQ, attention, visuospatial functioning, verbal and non-verbal learning and executive functioning (Mattson et al., 2001; Riley and McGee, 2005; Rasmussen et al., 2006; Fryer et al., 2007; Jacobson et al., 2008; Green et al., 2009; Crocker et al., 2011; Lewis et al., 2015; Lindinger et al., 2016). Investigation into the possible neuroanatomical changes that accompany this diagnosis is crucial as structural brain changes may underpin the observed functional deficits. A better understanding of brain changes may assist in planning more targeted interventions. The anatomical structures investigated in this dissertation were selected, in part, due to their association with some of the neurobehavioural deficits reported in children who have been prenatally exposed to alcohol.

The earliest evidence of effects of PAE on the brain were from autopsies (see review Spadoni et al., 2007). Neuroanatomical differences observed in children with PAE included hydrocephalus, porencephaly, increased cerebrospinal fluid (CSF), non-closure of the neural tube, developmental abnormalities in the prosencephalon, and anomalies in the cerebellum, hippocampus and corpus callosum (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et

al., 1983). Clarren and associates (1978) hypothesised that these changes were consequences of migration errors in neuronal and glial cells. Although autopsy studies are not ideal since only the most extreme cases of FAS result in death (Lebel et al., 2008), thus not reflecting the living brain (Spadoni et al., 2007), they did at the time provide evidence of PAE-related neuroanatomical alterations.

The timing, frequency and extent of alcohol exposure has been linked to the magnitude of damage and which structures are affected (see Guerri et al., 2009 for review). Many of the deficits seen in FAS are associated with craniofacial dysmorphology because both craniofacial and neuronal development have crucial changes at the embryonic stage of gastrulation (3-4 weeks into human pregnancy) (Guerri et al., 2009). Animal studies have shown that neuronal stem cells seem to be a target for the teratogenic effects of PAE during this stage of development, with even light exposure levels resulting in neuronal and behavioural deficits (Miller, 2007). At this critical stage, even a single high dose of alcohol to rodents results in caudatoputamen and ventromedial forebrain neuronal population deficits (Ashwell and Zhang, 1996). The second critical stage for PAE in human development is at gestational weeks 7-20 when neuroepithelial cells migrate and proliferate and the majority of the brain starts to differentiate (Guerri et al., 2009). Alcohol exposure at the equivalent stage has been shown to decrease neural progenitor cell proliferation in rodents, which results in reduced neuron and glial cells in areas such as the hippocampus (Miller, 1995; Nixon and Crews, 2002). Exposure to ethanol in the equivalent of the third trimester lead to long lasting deficits in the excitability of layer 5 pyramidal cells in the neocortex (Granato et al., 2012; Valenzuela et al., 2012). Despite these studies being performed on animal proxies and the animal gestation time periods being equated to human gestation, they do provide some insight into time- and dose-dependent alcohol effects on the brain with precise methods that cannot be performed on human participants.

Advancements in methods to investigate the brain mean that it can now be studied *in vivo*. Neuroimaging is non-invasive and a wide range of imaging modalities exist, each able to investigate different properties of the brain in a more targeted fashion (Spadoni et al., 2007). For example, brain imaging studies have supported animal research, which indicated that PAE does not appear to affect all cells of the cerebrum equally - white matter (WM) is seemingly more severely affected than grey matter (GM) on a range of imaging modalities (Archibald et al., 2001; Lebel et al., 2011; Donald et al., 2015). This result could be due to an

adverse effect of prenatal alcohol consumption on the process of myelination or developmental delay in the myelination process resulting in dysmorphology in WM structures, like the corpus callosum (Guerri, 2001).

The corpus callosum (CC) has been shown to be particularly vulnerable to PAE as evidenced by hypoplasia on mid-sagittal MRI scans (Mattson et al., 1992; Sowell et al., 2001; Autti-Rämö et al., 2007; Biffen et al., 2017) and disorganised fibre tracts or myelination disturbances (Bookstein et al., 2002; Lebel et al., 2008; Sowell et al., 2008). Many recent diffusion tensor imaging (DTI) studies have highlighted how microstructural changes in white matter associated with FASD and PAE are linked to various cognitive deficits (Ma et al., 2005; Lebel et al., 2008, 2011; Sowell et al., 2008; Fryer et al., 2009; Wozniak et al., 2009, 2011; Fan et al., 2016). These studies emphasize that understanding the communication backbone of the brain (white matter) is crucial to understanding various cognitive deficits associated with PAE. Structurally, the CC has been one of the most researched structures in the PAE literature (Riley and McGee, 2005; Lebel et al., 2012; Donald et al., 2015). Morphology appears to be related to function, with a thicker corpus callosum correlated to deficits in executive function, while a thinner morphology was associated with motor deficits (Bookstein et al., 2002). Further, tests of inter-hemispheric transfer have shown that children prenatally exposed to alcohol have deficits in inter-hemispheric transfer of tactile information (Roebuck et al., 2002; Dodge et al., 2009). Inter-hemispheric transfer can be investigated with finger localization tasks (FLT), which involve stimulating the tip of a finger (or series of fingers) and asking the participant to report back on which finger(s) were touched (Pipe, 1991). It has been used to investigate callosal function in both normal and clinical samples (Quinn and Geffen, 1986; Pipe, 1991; Roebuck et al., 2002; Dodge et al., 2009). FLT has previously been used to investigate the effects on prenatal alcohol exposure on inter-hemispheric transfer in children and young adults (Roebuck et al., 2002; Dodge et al., 2009). Both studies found alcohol-related deficits in interhemispheric transfer in children and that reduced CC size was related to poorer FLT performance. While Roebuck et al. (2002) found deficits in children with PAE, Dodge et al. (2009), however, reported deficits only in the most severely affected children with a diagnosis of FAS. Further, in young adults from the Detroit Longitudinal Cohort, Dodge et al. (2009) did not detect group differences but rather an association of interhemispheric transfer deficits with the amount of alcohol consumed by mothers per drinking occasion. As such, it remains to be established whether inter-

hemispheric transfer deficits are dose-dependent or a diagnostic threshold effect, and to what extent deficits reported in pre-adolescence persist into adolescence.

Also of interest to this dissertation is the effect of PAE on the hippocampal formation (including the hippocampus proper, dentate gyrus and subiculum) – often referred to collectively as the hippocampus. Many rat studies have focused on the effects of PAE on the hippocampus (Ikonomidou, 2000; Ikonomidou et al., 2001; Nixon et al., 2004), yet fewer studies, with more conflicting results, have investigated human subjects. An MRI study on children found that structural abnormalities of the hippocampus occur in specific individuals prenatally exposed to alcohol, rather than finding a within-group effect (Autti-Rämö et al., 2007). Some studies suggested that the left hippocampus was more vulnerable to PAE than the right hippocampus, as it showed reduced volume in individuals with FAS and FASD (Riikonen et al., 2007; Willoughby et al., 2008). Further, in the control individuals, the hippocampus increased in volume with age, however, this trend was not present in individuals with FASD, suggesting a long term effect of PAE on the development of the hippocampus (Willoughby et al., 2008). In contrast, another study, found the hippocampus significantly spared despite general hypoplasia in the cerebrum (Archibald et al., 2001). Neuropsychological studies have found certain functional deficits which correspond to characteristic functions of the hippocampus (Baars and Gage, 2010; Devinsky and D'Esposito, 2004; Maguire et al., 2006, 2000; Willoughby et al., 2008). Thus, it is hypothesised that certain structural differences may exist in the hippocampus based on these functional deficits, which motivated the investigation of this structure in this dissertation.

Further, there is a close link between structural differences in the basal ganglia and how they moderate functional outcomes in individuals with FAS (Archibald et al., 2001; Mattson et al., 1996; Spadoni et al., 2007). MRI studies have indicated that there is a decrease in the overall volume of the basal ganglia in individuals with FAS (Archibald et al., 2001; Mattson et al., 1996, 1992; S. N. Mattson et al., 1994). Caudate volume has been shown to be associated with neuropsychological test outcomes measuring verbal learning and recall, as well as inhibition (Mattson et al., 2001b). Mattson and colleagues (1996) found a lower volume in the basal ganglia of children with FAS. When the decrease in cerebral vault was controlled for and the basal ganglia divided into the caudate and lenticular nuclei, the caudate nucleus remained significantly and disproportionately decreased in volume. This finding was supported by a larger MRI study by Archibald and associates (2001), where the caudate

nucleus was again found to be disproportionately reduced in volume. Studies looking at the effect of PAE on basal ganglia volumes have also reported that the nucleus accumbens was reduced in volume, but only proportionately to the overall cerebral hypoplasia (Archibald et al., 2001; Mattson et al., 1996). This suggests that it may be relatively spared in these individuals. Relatively few studies have investigated the effects of FAS on the nucleus accumbens and how they may apply to function. Due to the protocol used when manually tracing the caudate nucleus (Cortese et al., 2006), the two structures can be seen to be very closely related and should be investigated simultaneously.

Despite advances in brain imaging allowing for detailed structural information, how volumetric differences are measured from these images can greatly influence the findings. Interpretation of brain imaging modalities such as structural MRI relies on valid and reliable objective measurement methods. For example, while it is well established that PAE has an effect on WM (eg. Archibald et al., 2001; Bookstein et al., 2002; Lebel et al., 2008; Fryer et al., 2009; Donald et al., 2015), the literature on GM is more divided (Lebel et al., 2011; Nardelli et al., 2011; Donald et al., 2015). Different methods of measuring volume may be one of the reasons for discrepancies in the literature, with potential differences between manual tracing and automated segmentation as well as between different automated segmentation programmes and versions. Further, automated segmentation protocols are often based on healthy adults from Western population groups, so it is sometimes uncertain how reliably and accurately these automated protocols can segment the brains of a clinical child population in another, less affluent population (Dewey et al., 2010; Morey et al., 2009). Manual tracing of the MRI scans is considered the gold standard, however, this method is time consuming, labour intensive and most accurate when a single neuroanatomical expert traces the entire sample (Morey et al., 2009). Yet, an increase in scan acquisition rates, as well as advances in time-saving analysis techniques in many fields of medical science has created a demand for automated segmentation of MRI scans.

Manual tracing is considered a well validated method in volumetric measurement, as expert neuroanatomists can employ heuristic protocols in smaller, more complex structures, like the hippocampus that automated programmes may struggle to delineate (Morey et al., 2009). Yet, in some structures, such as the CC, very different tracing protocols may be employed between manual and automated methods. Understanding how accurate automated segmentation is may assist in accounting for differences between segmentation methods and

may result in more convergent literature and improved automated segmentation methods. This highlights the importance of determining the actual differences between automated segmentation and manual tracing in neuroimaging studies, especially in different populations and at different ages, as well as understanding the limitations of each method with respect to measuring neuroantomical structures. Since comparison of different automated segmentation softwares found that FreeSurfer showed greater volume overlap, smaller volume difference and higher correlation estimates with manual tracing than other segmentation programs (Morey et al., 2009; Tae et al., 2008; Wenger et al., 2014), it was used for this dissertation.

In summary, the neurological effects of PAE are multifarious and result in the cognitive, behavioural and structural deficits associated with FASD (Ikonomidou, 2000; Olney, 2004; Hoyme et al., 2005; Spadoni et al., 2007; Lebel et al., 2008, Mattson et al., 1998; Astley et al., 2009). The high prevalence rates of FASD in high-risk communities from the Western Cape province of South Africa (May et al., 2000; Viljoen et al., 2005) warrants investigation into the neurological effects of PAE in this population. This dissertation had three primary aims: (1) to examine differences by diagnostic group in volumes of selected neuroanatomical structures from structural MRI via manual segmentation and potential associations of degree of alcohol exposure with brain volume reductions; (2) to investigate whether there were differences between the volumetric data from manual tracing and automated segmentation and how this would be reflected in a clinical sample; and (3) to investigate whether alcohol-related deficits in inter-hemispheric transfer of tactile information are observed in adolescence and the potential role of CC reductions in such deficits.

Motivated by previous structural and neuropsychological studies, it was hypothesised that there would be structural differences between children exposed prenatally to alcohol and controls in the CC, the hippocampus, and the caudate nucleus and nucleus accumbens of the basal ganglia. It was further hypothesized that PAE and smaller CC size would both be related to poorer performance on a finger localization task.

Chapter 2: Reductions in Corpus Callosum Volume Partially Mediate Effects of Prenatal Alcohol Exposure on IQ

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Abstract

Disproportionate volume reductions in the basal ganglia, corpus callosum and hippocampus have been reported in children with prenatal alcohol exposure (PAE). However, few studies have investigated these reductions in high prevalence communities, such as the Western Cape Province of South Africa, and only one study made use of manual tracing, the gold standard of volumetric analysis. The present study examined the effects of PAE on subcortical neuroanatomy using manual tracing and the relation of volumetric reductions in these regions to IQ and performance on the California Verbal Learning Test-Children's Version (CVLT-C), a list learning task sensitive to PAE. High-resolution T1-weighted images were acquired, using a sequence optimized for morphometric neuroanatomical analysis, on a Siemens 3T Allegra MRI scanner from 71 right-handed, 9- to 11-year-old children (9 fetal alcohol syndrome (FAS), 19 partial FAS, 24 non-syndromal heavily exposed (HE) and 19 non-exposed controls). Frequency of maternal drinking was ascertained prospectively during pregnancy using timeline follow-back interviews. PAE was examined in relation to volumes of the corpus callosum (CC) and left and right caudate nuclei, nucleus accumbens and hippocampi. All structures were manually traced using Multitracer. Higher levels of PAE were associated with reductions in CC volume after adjustment for TIV. Although the effect of PAE on CC was confounded with smoking and lead exposure, additional analyses showed that it was not accounted for by these exposures. Amongst dysmorphic children, smaller CC was associated with poorer IQ and CVLT-C scores and statistically mediated the effect of PAE on IQ. In addition, higher levels of PAE were associated with bilateral volume

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reductions in caudate nuclei and hippocampi, effects that remained significant after control for TIV, child sex and age, socioeconomic status, maternal smoking during pregnancy, and childhood lead exposure. These data confirm previous findings showing that PAE is associated with decreases in subcortical volumes and is the first study to show that decreases in callosal volume may play a role in fetal alcohol-related impairment in cognitive function seen in childhood.

2.1 Introduction

Prenatal alcohol exposure (PAE) is associated with a range of neurocognitive and behavioural problems. Fetal alcohol spectrum disorders (FASD) is an umbrella term under which the entire spectrum of outcomes related to PAE falls. Fetal alcohol syndrome (FAS), the most severe of the fetal alcohol spectrum disorders (FASD), is characterized by growth deficits, small head circumference, distinctive craniofacial dysmorphology (small palpebral fissures, flattened philtrum and thin vermillion) and cognitive problems that relate to specific brain abnormalities (Hoyme et al., 2005a; Jones and Smith, 1973). Partial FAS (PFAS) is diagnosed in individuals exhibiting the facial dysmorphology characteristic of FAS, whose mothers are known to have drunk heavily during pregnancy and who exhibit growth deficits, small head circumference or neurobehavioral impairment (Hoyme et al., 2005).Adverse effects on brain development range from microstructural, neurochemical and cellular dysfunction (such as widespread apoptotic neurodegeneration that can result in the death of millions of cells in the forebrain during gestation) to gross structural abnormalities (Ikonomidou, 2000; Olney, 2004; Spadoni et al., 2007; Lebel et al., 2008).

In the U.S. and in Western countries, FAS occurs at a rate estimated to be between 0.05 to 7 per 1000 live births (May and Gossage, 2001; May et al., 2009). In contrast, certain high-risk communities in South Africa have been found to have an FAS prevalence rating that is 18 to 148 times greater than in the U.S. (May et al., 2000; Urban et al., 2008; Viljoen et al., 2005). Within the Western Cape province of South Africa, rates are highest amongst the Coloured population of mixed Asian, African and European ancestry (May et al., 2013b, 2005, 2000; Viljoen et al., 2005).

Magnetic resonance imaging (MRI) allows for non-invasive and quantitative investigation of the structural and functional brain alterations that are observed in FASD. Brain regions that

have been consistently implicated in FASD include the corpus callosum (CC), cerebellum, parietal lobe, frontal lobe, temporal lobe, caudate nucleus, hippocampus and increased volume of lateral ventricles (see review papers, Lebel et al., 2011; Donald et al., 2015). While most regions show alcohol-related volume reductions, findings in certain subcortical brain regions have not always been consistent. For example, some studies report bilateral decreases in hippocampal volume (Willoughby et al., 2008; Astley et al., 2009; Coles et al., 2011; Nardelli et al., 2011; Treit et al., 2013) with PAE, while others find relative sparing (Archibald et al., 2001) or even volume increases (Riikonen et al., 2007). Methodological differences, such as automated versus manual segmentation, failure or different strategies to control for total intracranial volume (TIV), small sample sizes, or large age ranges may explain some of these differences. Notably, one study found increased hippocampal volume relative to brain volume with age (9-15 yr) in controls, but not in children with FASD (Willoughby et al., 2008), highlighting the potential contribution of age-related changes in brain development when comparing diagnostic groups and the need for narrow age ranges. It is not yet known to what extent developmental trajectories in children prenatally exposed to alcohol are altered.

Although manual tracing is considered the gold standard for volumetric analysis, yielding the greatest consistency and sensitivity (Morey et al., 2009), few studies employ this method due to high time demands (Archibald et al., 2001; Riikonen et al., 2007; Willoughby et al., 2008; Astley et al., 2009). Here we use manual tracing of study participants within a narrow age range (10.4 ± 0.4 yr) to examine the effects of PAE on volumes of select subcortical structures and the CC in 81 children from the Cape Town Longitudinal Cohort (Sandra W Jacobson et al., 2008), for whom diagnostic and detailed prospective prenatal alcohol and drug exposure data are available. Most previous studies have only been able to examine volumetric differences between diagnostic groups and not dose-dependent effects, since the amount of alcohol used during pregnancy is difficult to recall reliably in retrospective case-controlled studies (Jacobson et al., 2002).

Due to the labour intensive nature of manual tracing, only subcortical structures previously implicated in FASD were investigated (Archibald et al., 2001; Morey et al., 2009; Nardelli et al., 2011). These include the caudate nucleus and hippocampus, which are involved in cognition, memory and emotional networks (Devinsky and D'Esposito, 2004) that are amongst the most affected domains in FASD (Green et al., 2009; Jacobson et al., 2011; Lewis

et al., 2016; Mattson et al., 1998; Uecker and Nadel, 1996; Willoughby et al., 2008). Although findings in the nucleus accumbens have rarely been reported in FASD (Archibald et al., 2001), we report it here because the manual tracing protocol we used for the caudate initially involved tracing both structures together. The CC was included as it is clearly visible on MRIs, is easy to delineate, and has been consistently shown to be reduced in FASD (Mattson et al., 1992; Sowell et al., 2001, 2008; Bookstein et al., 2002; Autti-Rämö et al., 2007; Lebel et al., 2008; Astley et al., 2009), even in newborns (Jacobson et al., 2017). PAE has been linked to a larger angle of the splenium (Bookstein et al., 2007), reductions in CC thickness and area in the splenium and anterior third (Yang et al., 2012), and CC volume reductions in the splenium, genu and area just anterior to the splenium (Riley et al., 1995).

In addition, we examined whether alcohol-related regional volumetric changes mediate the effect of PAE on cognitive performance on the Wechsler Intelligence Scale for Children-Fourth Edition (WISC-IV) and the California Verbal Learning Test-Children's Version (CVLT-C). Poorer IQ is frequently seen in FASD (Streissguth et al., 1990, 1991; Jacobson et al., 2004) as is impaired performance on the CVLT-C (Delis et al., 1994), a list learning task (Kerns et al., 1997; Mattson et al., 1998; Crocker et al., 2011; Vaurio et al., 2011; Suttie et al., 2013; Lewis et al., 2015).

We hypothesized that increasing PAE would be associated with volumetric reductions in all regions examined and that these effects would remain significant after controlling for reductions in total brain volume. We also hypothesized that volumetric reductions in hippocampus, caudate nucleus and CC would be associated with poorer WISC IQ and CVLT-C performance and that the effects of PAE on these outcomes would be partially mediated by volumetric changes in the regions that were traced.

2.2 Methods

2.2.1 Participants

From 1998-2002 pregnant women were recruited into the Cape Town Longitudinal Cohort (Jacobson et al., 2008) from an antenatal clinic in a Cape Coloured community in Cape Town known to have a high prevalence of alcohol consumption (Croxford and Viljoen, 1999). Detailed alcohol exposure data collected prospectively during pregnancy were available for all of these children. Mothers were interviewed at enrolment and at two subsequent visits using the timeline follow-back (TLFB) approach to record alcohol consumption during pregnancy (Jacobson et al., 2002). We adapted the interview to include information about the type of beverage consumed, container size (including pictures of different containers, bottles, cans, glass size) and sharing (size of container divided by number of women drinking together) to reflect how pregnant women in this community tend to drink. The mother was asked about her drinking on a day-by-day basis during a typical 2-week period around the time of conception, with recall linked to specific times of day and activities. If her drinking had changed since conception, she was also asked about her drinking during the past 2 weeks and when her drinking had changed. At the two follow-up visits, the mother was again asked about her drinking during the previous 2 weeks. Volume was recorded for each type of alcohol beverage consumed each day and converted to oz of absolute alcohol (AA) using multipliers proposed by Bowman et al. (1975) (liquor—0.4, beer—0.05, wine—0.12, cider—0.06). Six summary measures were constructed—average oz AA per day at conception, AA per day averaged across pregnancy, AA per drinking day (quantity per occasion) at conception and across pregnancy, and number of drinking days per week (frequency) at conception and across pregnancy.

Any woman who reported drinking at least 1.0 oz AA per day (≈ 2 standard drinks per day) or at least 2 instances of binge drinking (≥5 drinks per occasion) during the first trimester was invited to participate. Women initiating antenatal care who reported no binge drinking and minimal alcohol use (<0.5 oz AA/ day) were invited to participate as controls. In this sample all but one of the mothers in the control group reported abstaining from drinking during pregnancy; the single control mother who consumed only 2 drinks/occasion on 2-3 days during the pregnancy. Smoking was recorded as the number of cigarettes smoked per day. Participants were excluded if they were less than 18 years of age, presented with chronic

medical problems including epilepsy, diabetes, HIV, or cardiac problems. Infant exclusionary criteria were major chromosomal anomalies, neural tube defects, multiple births, and seizures.

In 2005, we organized a clinic in which the children in the study were each independently examined by two expert dysmorphologists (E.H. Hoyme, M.D., and L.K. Robinson, M.D.) for growth and FAS related dysmorphic features using a standard protocol (Hoyme et al., 2005) based on the Revised Institute of Medicine criteria (Jacobson et al., 2008). Case conferences were held in which the dysmorphologists, SWJ, JLJ, and CDM determined which of the children met criteria for FAS or PFAS. Children who did not meet criteria for FAS of PFAS were designated as either non-syndromal heavily exposed (HE) or controls, depending on the maternal alcohol history. Five children who could not attend the 2005 clinic were examined by another expert FASD dysmorphologist (N. Khaole, M.D.), whose diagnoses were all subsequently confirmed by examinations conducted in follow-up clinics we held with the same dysmorphologists in 2009 and with HEH in 2013 and 2016.

2.2.2 Magnetic Resonance Image (MRI) Acquisition

MRI scans were acquired at the Cape Universities Brain Imaging Centre (CUBIC) using a 3T Siemens Allegra MRI scanner (Siemens Medical Systems, Erlangen, Germany) for 81 right-handed children from this cohort at age 9-11 years. High-resolution T1-weighted images were obtained using a volumetric navigated (Tisdall et al., 2012) multi-echo magnetization prepared rapid gradient echo (MEMPRAGE) sequence optimized for morphometric neuroanatomical analysis using FreeSurfer software (van der Kouwe et al., 2008). Imaging parameters were: FOV 256×256mm, 128 sagittal slices, TR 2530mm, TE 1.53/3.21/4.89/6.57mm, TI 1100ms, flip angle 7°, voxel size 1.3×1.0×1.3 mm^3, acquisition time 8:07 minutes. The volumetric navigator provides real-time motion tracking and correction to reduce artefacts resulting from motion.

Human subject participation was approved by the ethics committees of the University of Cape Town Faculty of Health Sciences and Wayne State University. Mothers provided written informed consent and children, oral assent. Examiners were blind with respect to PAE history and FASD diagnosis, except in the most severe cases of FAS. Total intracranial volumes (TIV) were obtained from FreeSurfer (Dale et al., 1999; Fischl et al., 2002a). Called

eTIV or ICV, within FreeSurfer, the programme calculates TIV by using an atlas scaling factor that it obtains from registering the images from the sample to an average template and using that scaling factor to estimate each participant's TIV (Buckner et al., 2004, see also http://surfer.nmr.mgh.harvard.edu/fswiki/eTIV).

2.2.3 Manual Tracing Protocol

The CC and left and right caudate nuclei, nucleus accumbens and hippocampi were manually traced on the MR images using Multitracer (Woods, 2003) software on a tablet laptop (Lenovo ThinkPad X200, Wacom digitizer pen enabled). A single blinded investigator performed all manual tracings (SCB). Tracing was supervised and reviewed by CW, the senior neuroanatomist at the University of Cape Town. All structures were traced in the coronal plane, with the exception of the CC that was traced in the sagittal plane. Orthogonal planes were used to inform border delineation. "Frust" volumes were recorded for each structure, which assumes that a contour applies to the centre of the slice on which it is drawn and that the square root of the cross-sectional area changes linearly across the slice thickness (Woods, 2003).

The CC (Fig. 2.1A) is clearly visible as a curved WM structure near the midline slice. The superior border is the cingulate gyrus and the CSF (cerebrospinal fluid) of the lateral and posterior fissure form the inferior border. The midline slices (about slices 127-130) were determined by locating the smallest area of the thalamus. Bookstein (2002) used the term "midline" to denote the slices located most medially in the corpus callosum (i.e. along the midline of the brain). The CC was traced on two contiguous midline slices. These were averaged and volume calculated.

The caudate nucleus (Fig. 2.1B) is a grey matter (GM) structure that lies on the lateral wall of the lateral ventricle and is traced until it disappears. The medial border is the lateral ventricle and the lateral border is formed by WM. The inferior border is the nucleus accumbens (Fig. 2.1C), which can only be differentiated histologically by cytoarchitecture, however, it can be removed from the caudate nucleus reliably by drawing a straight line from the inferior border of the lateral ventricle to the bottom-most border of the internal capsule. Laterally, it is separated from the putamen by a line descending from the internal capsule. It was traced until the anterior commissure bridges the midline.

The anterior border of the hippocampus (Fig. 2.1D) is the inner surface of the alveus. Tracing proceeds past the crura of the fornices until hippocampal GM disappears. The medial border includes the subiculum to the supramedial border of WM in the temporal lobe. The lateral border is the inner surface of the alveus within the temporal horn of the lateral ventricle. Inferiorly, the boundary is the interface between the WM and GM.

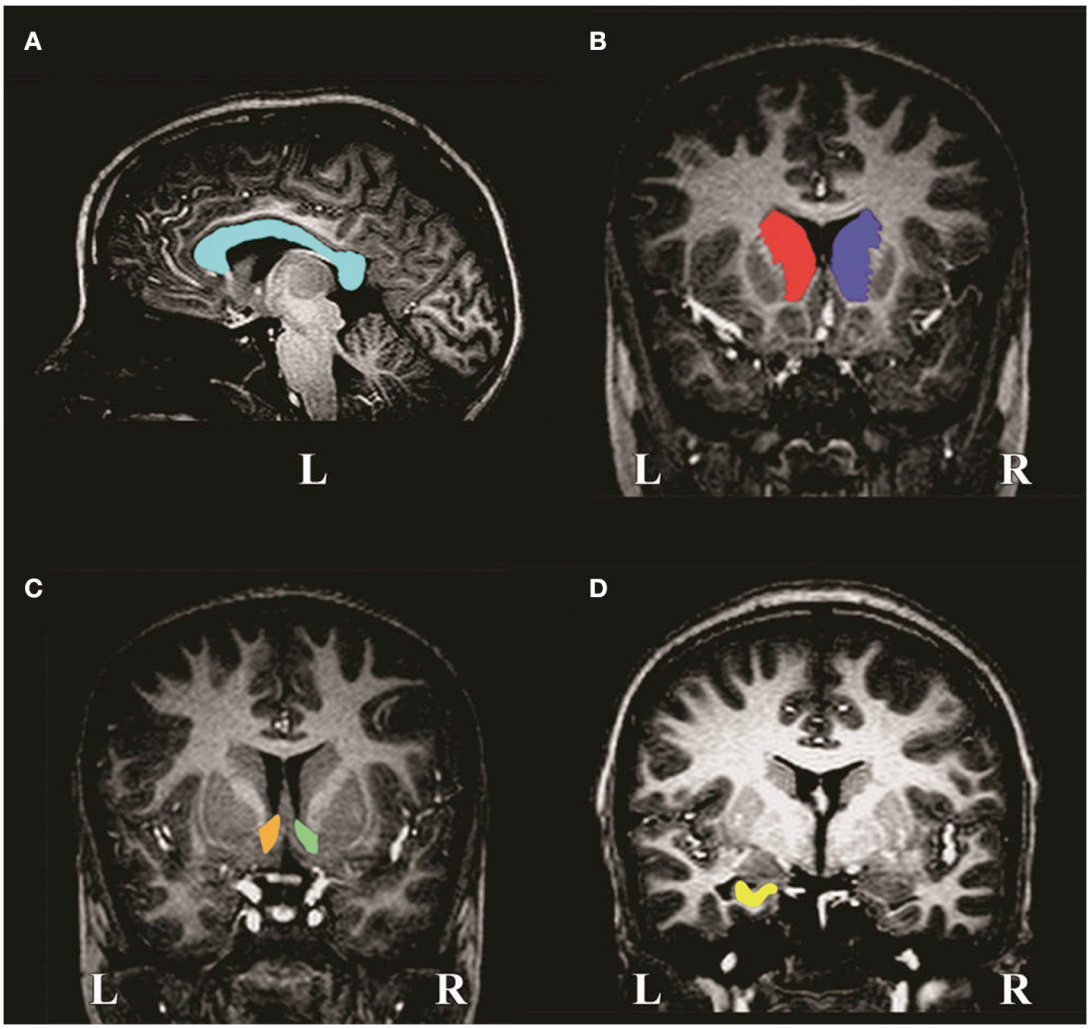

Figure 2.1: MR image slices of a non-exposed control child showing manual tracings of the (A) corpus callosum, (B) left and right caudate nuclei, (C) left and right nucleus accumbens and (D) left hippocampus. L and R denote left and right respectively.

Three participants (2 HE; 1 control) were excluded due to pathology (unrelated to the research question) and 7 (1 FAS; 2 HE; 4 control) due to compromised image quality. Pathology included one scan where there were hypointense inclusions on the superior surface of the right lateral ventricle (indicative of a potentially conflicting diagnosis), and two scans

where the lateral ventricles were occluded, making delineation of caudate nuclei and nucleus accumbens problematic.

2.2.4 Statistical Analyses

Analysis of variance (ANOVA) was performed for each region of interest (ROI) to examine whether structural volumes differed between diagnostic groups. Pearson correlation was used to examine the relations of ROI volumes to each of the potential confounders. Potential control variables included total intracranial volume (TIV), child's sex and age (years) at scan, socioeconomic status (SES; Hollingshead, 2011), maternal smoking during pregnancy (cigarettes/day), and postnatal lead exposure (ug/dl) obtained from a blood sample at 5 years. ANCOVA was used to examine whether volumetric group differences remained significant after controlling for confounders even weakly ($p < 0.10$) related to the outcome. Although marijuana (days/week) and cocaine (days/week) were considered, only 5 mothers in this sample reported marijuana use and 1 cocaine. Any observed between-group differences were, therefore, rerun excluding children born to these mothers to see whether the effects persisted without them.

The association between continuous measures of PAE (both at conception and across pregnancy) and structural volumes were examined using Pearson correlation. Hierarchical multiple regression analyses were used to control for confounders. The alcohol measure was entered in the first step of each analysis for each outcome. Total intracranial volume (TIV) was then added to the regressions for the regional volumes to determine if the exposure affected the regional volume over and above its effect on total brain volume. Control variables related to the outcome at less than $p < 0.10$ were entered in the third step to determine if the effect of the prenatal alcohol measure on structure volume continued to be significant after statistical adjustment for these potential confounders. Observed associations with extent of alcohol exposure were rerun excluding children born to mothers who used marijuana or cocaine during pregnancy to see whether effects remained without them.

Pearson correlations were used to examine whether alcohol-related volumetric changes were associated with performance on the WISC-IV IQ and CVLT-C, two outcomes known to be related to PAE. CVLT-C data were only available for 66 children. Mediation of effects of PAE on these cognitive outcomes by structural volumes associated with PAE was examined

using multiple regression. The effect of PAE on the cognitive outcome was entered in Step 1 of the regression; the structure volume, in Step 2. Mediation was inferred if the effect of PAE was significantly reduced when the structure volume was entered in Step 2, based on the Clogg test (Clogg et al., 1992).

2.3 Results

Following the exclusions described above, we report data for 71 children. Demographic characteristics are presented in Table 2.1. Although the FASD diagnostic groups did not differ by sex, prenatal exposure to cigarettes, marijuana, or cocaine, or childhood lead exposure, children in the HE group were slightly older than those in the FAS and PFAS groups. As expected, children in both the FAS and PFAS groups had lower IQs than the HE children; those in the PFAS group also had lower scores than the controls. Total brain volumes were smaller in the children with FAS compared to all other groups. SES was lower for mothers of children with FAS or PFAS than mothers of children from the HE and control groups.

As expected, all of the alcohol measures for the control group were statistically lower compared to the alcohol-using groups (Table 2.1). In addition, children with FAS were more exposed than HE children on all measures. They were also exposed to more alcohol/drinking day at conception than those in the PFAS group. At conception and across pregnancy, AA/day and drinking days/week for the PFAS group were also significantly higher than for the HE group. Although number of days of alcohol use was reduced from 1.5 (at conception) to 1.1 (across pregnancy) day/week on average ($t(68)=3.88$, $p<0.001$), the number of drinks/occasion remained virtually unchanged, ($t(69)=-0.33$, $p=0.741$). Moreover, alcohol consumption at conception was highly correlated with alcohol use throughout pregnancy ($r=0.923$). Due to the small sample size of the FAS group, the two syndromal groups, FAS and PFAS, were combined for subsequent volumetric analyses.

Table 2.1: Sample characteristics (N=71)

		Control		Exposed HE		PFAS		FAS		TOTAL		*F* or X^2
		(*n*=19)		(*n*=24)		(*n*=19)		(*n*=9)		(*N*=71)		
Child:												
Sex: Male	*n*(%)	9	(47%)	15	(63%)	11	(58%)	4	(44%)	39	(55%)	1.46
Age (yr) at scan[a]	Mean(SD)	10.6	(0.5)	11.0	(0.7)	10.5	(0.4)	10.4	(0.9)	10.7	(0.7)	2.84*
WISQ-IV IQ[b]	Mean(SD)	73	(10.7)	76	(11.3)	64	(10.2)	65	(13.1)	71	(12.1)	5.44**
Total intracranial volume ($x10^6$ mm^3)[c]	Mean (SD)	1.3	(0.1)	1.4	(0.2)	1.3	(0.1)	1.2	(0.1)	1.3	(0.1)	3.24*
Maternal:												
Socioeconomic status[d]	Mean(SD)	23.5	(1.9)	24.0	(1.7)	15.2	(1.5)	16.9	(3.6)	20.6	(9.0)	5.44**
Cigarettes/day	Median(IQR)	0.0	(5.0)	5.3	(7.8)	7.5	(7.0)	9.0	(5.5)	5.0	(10.0)	1.34
Marijuana (yes)	*n*(%)	0	(0%)	2	(8%)	2	(11%)	1	(11%)	5	(7%)	2.08
Cocaine (yes)	*n*(%)	0	(0%)	0	(0%)	1	(5%)	0	(0%)	1	(1%)	2.78
Lead (ug/dl)	Mean(SD)	9.2	(3.5)	9.3	(3.3)	11.5	(6.5)	11.7	(4.3)	10.2	(4.6)	1.47
Alcohol at conception												
AA/day (oz)[e]	Median(IQR)	0.0	(0.0)	0.5	(1.0)	1.2	(1.2)	1.4	(1.6)	0.6	(1.3)	26.52***
AA/drinking day (oz)[f]	Mean(SD)	0.06	(0.3)	2.8	(2.2)	4.0	(2.1)	5.7	(2.8)	2.8	(2.7)	21.33***
Drinking days/wk[g]	Mean(SD)	0.0	(0.1)	1.3	(1.1)	2.6	(1.4)	2.8	(1.9)	1.5	(1.6)	18.88***
Alcohol across pregnancy												
AA/day (oz)[h]	Median(IQR)	0.0	(0.0)	0.2	(0.7)	1.0	(0.6)	0.8	(1.8)	0.3	(1.0)	22.73***
AA /drinking day (oz)[i]	Mean(SD)	0.06	(0.3)	3.4	(2.4)	3.8	(1.7)	4.9	(1.8)	2.8	(2.5)	21.83***
Drinking days/wk[j]	Mean(SD)	0.0	(0.0)	1.0	(0.8)	2.0	(1.0)	2.1	(1.8)	1.1	(1.2)	17.90***

For skewed data medians and interquartile ranges (IQR) were used. HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS; WISC-IV Wechsler Intelligence Scales for Children-Fourth Edition

[a] HE > FAS, PFAS (both $p<0.05$)

[b] FAS < HE ($p=0.011$); PFAS < HE, Control (both $p\leq0.01$)

[c] FAS < PFAS ($p=0.018$), HE ($p=0.003$), Control ($p=0.038$)

[d] Hollingshead, 2011; FAS < HE, Control (both $p\leq0.05$); PFAS < HE, Control (both $p<0.01$)

[e] Control < FAS, PFAS, HE (all $p<0.001$); HE < FAS, PFAS (both $p<0.001$)

[f] Control < FAS, PFAS, HE (all $p<0.001$); HE < FAS ($p<0.001$); PFAS < FAS ($p=0.027$)

[g] Control < FAS, PFAS, HE (all $p\leq0.001$); HE < FAS, PFAS (both $p<0.01$)

[h] Control < FAS, PFAS, HE (all $p<0.001$); HE < FAS, PFAS (both $p\leq0.001$)

[i] Control < FAS, PFAS, HE (all $p<0.001$); HE < FAS ($p=0.036$)

[j] Control < FAS, PFAS, HE (all $p\leq0.001$); HE < FAS, PFAS (both $p<0.01$)

*$p\leq0.05$, **$p\leq0.01$,

***$p\leq0.001$

Volumes of traced regions are reported for each diagnostic group in Table 2.2 and associations of control variables with structural volumes in Table 2.3. Children in the FAS/PFAS group had smaller right hippocampi compared to both the HE and control children, but this effect was no longer significant after adjustment for TIV. Volumes of four regions were smaller in girls than in boys. Among the remaining potential confounders, smoking during pregnancy and postnatal lead exposure were related to size in one region—the CC.

Table 2.2: Mean and standard deviations of structure volumes by diagnostic group

ROI	Control (*n*=19)	HE (*n*=24)	FAS/PFAS (*n*=28)	Total (*N*=71)	*p*	p^1	p^2
Grey matter ROIs							
L Caudate [a]	3929 (563)	3798 (543)	3581 (563)	3748 (567)	0.10	0.11	0.11
R Caudate [a]	3936 (582)	3804 (552)	3662 (607)	3783 (584)	0.29	0.26	0.24
L NA [a]	542 (117)	549 (72)	503 (89)	529 (93)	0.16	0.37	0.28
R NA [a]	490 (95)	490 (83)	467 (90)	481 (88)	0.57	0.78	0.44
L Hippocampus	2400 (224)	2392 (346)	2277 (360)	2349 (325)	0.33	0.53	0.53
R Hippocampus	2432 (185)	2469 (302)	2266 (318)	2379 (294)	**0.03**[#]	0.09	0.09
White matter ROI							
CC [b,c]	564 (64)	570 (107)	525 (79)	551 (88)	0.13	0.21	0.52

Values are mean (SD). Volumes are in mm^3. NA nucleus accumbens; CC corpus callosum; HE heavily exposed non-syndromal; FAS fetalalcohol syndrome; PFAS partial (FAS)

Bold print denotes significance at $p \leq 0.05$

[#]FAS/PFAS<HE non-syndromal (p=0.012) and Control (p=0.052)

p^1 after controlling for TIV

p^2 after controlling for TIV, as well as (a) sex of child, (b) cigarettes/day during pregnancy, (c) lead concentration (ug/dl)

Table 2.3: Pearson correlations of control variables with structure volumes

ROI (mm^3)	TIV	Sex	Cigarettes /day during pregnancy	Socioeco-nomic status	Lead concentration (ug/dl)	Age (yr)
Grey matter ROIs						
L Caudate	**0.520**	**-0.215**	-0.113	0.087	-0.168	-0.082
	(0.001)	**(0.079)**	(0.350)	(0.468)	(0.161)	(0.499)
R Caudate	**0.496**	**-0.222**	-0.066	0.057	-0.126	-0.069
	(0.001)	**(0.052)**	(0.584)	(0.638)	(0.296)	(0.565)
L NA	**0.421**	**-0.367**	0.004	0.061	0.120	-0.080
	(0.001)	**(0.002)**	(0.975)	(0.614)	(0.321)	(0.505)
R NA	**0.504**	**-0.381**	0.041	-0.012	0.148	-0.081
	(0.001)	**(0.001)**	(0.735)	(0.920)	(0.218)	(0.502)
L Hippocampus	**0.349**	-0.137	-0.109	-0.068	-0.141	0.058
	(0.003)	(0.255)	(0.364)	(0.574)	(0.242)	(0.630)
R Hippocampus	**0.402**	-0.167	-0.076	-0.059	-0.043	0.016
	(0.002)	(0.307)	(0.526)	(0.625)	(0.719)	(0.892)
White matter ROI						
CC	0.184	0.043	**-0.325**	0.166	**-0.209**	0.087
	(0.124)	(0.723)	**(0.006)**	(0.166)	**(0.080)**	(0.470)

Bold print denotes significance at $p \leq 0.1$

Values are Pearson *r* (*p*)

NA nucleus accumbens; CC corpus callosum

Sex (male=1, female=2)

Table 2.4: Association of alcohol consumption measures with structure volumes

ROI	Absolute alcohol/day			Drinking days/week		
	r (*p*)	β_1 (*p*)	β_2 (*p*)	*r* (*p*)	β_1 (*p*)	β_2 (*p*)
Grey matter ROIs						
L Caudate [a]	**-0.366 (0.002)**	**-0.291 (0.005)**	**-0.366 (0.001)**	**-0.399 (0.001)**	**-0.317 (0.002)**	**-0.400 (<0.001)**
R Caudate [a]	**-0.311 (0.008)**	**-0.241 (0.023)**	**-0.311 (0.007)**	**-0.333 (0.005)**	**-0.256 (0.016)**	**-0.334 (0.004)**
L NA [a]	-0.142 (0.239)	-0.090 (0.404)	-0.142 (0.204)	-0.173 (0.149)	-0.117 (0.279)	-0.175 (0.117)
R NA [a]	-0.085 (0.481)	-0.016 (0.878)	-0.085 (0.446)	-0.115 (0.341)	-0.039 (0.711)	-0.116 (0.298)
L Hippocampus	**-0.295 (0.012)**	**-0.245 (0.031)**	**-0.245 (0.031)**	**-0.313 (0.008)**	**-0.257 (0.024)**	**-0.257 (0.024)**
R Hippocampus	**-0.275 (0.020)**	**-0.215 (0.054)**	**-0.215 (0.054)**	**-0.281 (0.018)**	-0.214 (0.056)	-0.214 (0.056)
White matter ROI						
CC [bc]	**-0.335 (0.004)**	**-0.313 (0.008)**	-0.179 (0.176)	**-0.353 (0.003)**	**-0.330 (0.005)**	-0.206 (0.116)

Bold print denotes significance at $p \leq 0.05$

β_1 is the standard regression coefficient adjusted for TIV

β_2 is the standard regression coefficient controlling for TIV, as well as (a) sex of child, (b) cigarettes/day during pregnancy, (c) lead concentration (ug/dl)

Table 2.4 presents the results of the multiple regression analyses examining the relation of continuous measures of PAE to the structural volumes. PAE was associated with volume reductions bilaterally in the caudate and hippocampus and smaller CC, but the effect on the CC was no longer significant after controlling for smoking during pregnancy and postnatal

lead exposure. When the regression for CC was rerun controlling only for lead, the effect of absolute alcohol/day remained significant (β = -0.281, p =0.022). In a regression of the effect of alcohol and smoking on CC volume, the effects of both of these exposures fell just short of significance (alcohol β = -0.236, p = 0.065; smoking β = -0.217, p = 0.089), suggesting that neither effect was attributable to the effect of the other exposure.

Table 2.5: Associations of structure volumes with IQ and CVLT-C scores

ROI	IQ				CVLT-C			
	Control *n*=19	HE *n*=24	FAS/PFAS *n*=28	Total *N*=71	Control *n*=19	HE *n*=22	FAS/PFAS *n*=25	Total *N*=66
L Caudate	-0.064	-0.190	0.164	0.081	0.112	-0.359	0.045	-0.013
	(0.795)	(0.375)	(0.405)	(0.504)	(0.647)	(0.101)	(0.832)	(0.916)
R Caudate	-0.118	-0.118	0.128	0.050	0.065	-0.232	0.098	-0.021
	(0.629)	(0.584)	(0.516)	(0.682)	(0.791)	(0.299)	(0.641)	(0.870)
L Hippocampus	0.136	-0.069	0.032	0.087	0.089	-0.286	**-0.439**	-0.233
	(0.580)	(0.749)	(0.871)	(0.469)	(0.716)	(0.196)	**(0.028)**	(0.060)
R Hippocampus	-0.064	-0.088	-0.125	0.055	0.251	-0.205	**-0.472**	-0.172
	(0.794)	(0.683)	(0.527)	(0.646)	(0.300)	(0.361)	**(0.017)**	(0.168)
CC	-0.122	0.324	**0.381**	**0.327**	-0.060	0.001	**0.389**	-0.060
	(0.619)	(0.122)	**(0.045)**	**(0.005)**	(0.808)	(0.997)	**(0.049)**	(0.808)

NA nucleus accumbens; CC corpus callosum; HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial (FAS)

Bold print denotes significance at $p \leq 0.05$

Values are Pearson r (p)

PAE was associated with lower IQ (r=-0.402; p=0.001) and CVLT-C (r=-0.248; p=0.045) scores. Larger CC was associated with higher IQ for the sample as a whole and with better CVLT-C performance for the FAS/PFAS group (Table 2.5). Unexpectedly, smaller hippocampi were associated with better CVLT-C scores for the FAS/PFAS group.

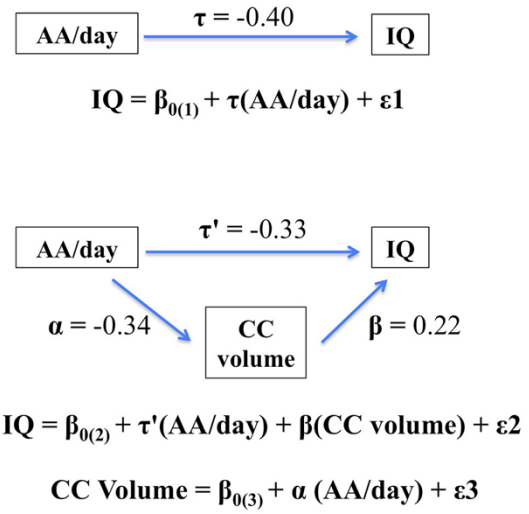

Figure 2.2: Path model showing partial mediation of the association between AA/day and IQ by corpus callosum (CC) volume. The figure shows that the effect of prenatal alcohol exposure on IQ is partially mediated by the fetal alcohol-related corpus callosum volume reduction. When CC size was added in Step 2 of the regression analysis, the effect of AA/day on IQ was reduced from -0.40 to -0.33, a reduction that was statistically significant, Clogg (t=-2.86, p<0.01).

The hypothesis that CC size would mediate the effect of PAE on IQ was tested using multiple regression. PAE was entered in Step 1. When CC size was added in Step 2 of the regression analysis, the effect of AA/day on IQ was reduced from -0.40 to -0.33, a reduction that was statistically significant, Clogg (t=-2.86, p<0.01). These data thus indicate that the effect of PAE on IQ was partially mediated by the alcohol-related reduction in CC size (Fig. 2.2).

2.4 Discussion

This study shows that increasing PAE, both in terms of quantity and frequency, is associated with disproportionate volume reductions bilaterally in the caudate nuclei and hippocampi and smaller CC. By contrast, volume differences between FASD diagnostic groups were evident only in the right hippocampus. Smaller CC was associated with poorer IQ. Within the FAS/PFAS group, smaller CC was also associated with lower CVLT-C scores, but hippocampal volume reductions were associated with better CVLT-C performance. The results from our mediation analysis suggest that the adverse effect of PAE on IQ is partially attributable to the reductions in CC size.

When the regional volumetric measures were compared across the three diagnostic groups, no significant differences were seen after adjustment for TIV. By contrast, the continuous measures of PAE were associated with volumetric differences in all of the regions examined after adjustment for brain size, except the bilateral NA. These findings are consistent with other neuroimaging studies performed with children from the same Cape Town cohort, which found that continuous measures of PAE were more sensitive than diagnosis to fetal alcohol-related alterations in brain structure and function (De Guio et al., 2014; du Plessis et al., 2015; Meintjes et al., 2014).

Our finding of alcohol-related volumetric reductions bilaterally in the caudate nuclei are consistent with studies reporting decreased basal ganglia volume in FAS (Mattson et al., 1994b, 1996, 1999; Archibald et al., 2001; Mattson et al., 2001; Roussotte et al., 2012). Animal studies suggest that the physiological mechanisms underlying smaller striatal volumes may be explained by a reduction in dendritic tree formation rather than a reduction in number of parvalbumin interneurons (De Giorgio et al., 2012) and may also be influenced by apoptosis of the main population (medium spiny neurons) within the caudate nucleus (Ikonomidou, 2000). The caudate nucleus is involved in many of the neurobehavioural deficits exhibited by children prenatally exposed to alcohol, including executive function domains, such as cognitive flexibility, concept formation and reasoning, planning and response inhibition (Mattson et al., 2001b, 1999). This region has also been shown to be activated less by children with FASD during behavioural inhibition tasks (Fryer et al., 2007) and to mediate cognitive control and verbal learning in children with PAE (Fryer et al., 2012). Although the latter study reported that larger caudate nuclei were associated with better performance in verbal learning in alcohol-exposed children, we did not confirm that finding in our study.

The caudate nuclei may be more susceptible to the effects of PAE than the NA. It has been suggested that some midline brain structures are more affected by alcohol exposure during embryological development than others (Meintjes et al., 2014; Sulik et al., 1981; Zhou et al., 2003). Our results are consistent with those of Archibald and associates (2001), who also failed to find alcohol-related volume reductions in the NA using a similar tracing methodology. Because the NA can only truly be differentiated from the caudate nucleus histologically, the lack of association in both these studies may be due to poor sensitivity by these tracing methods to differentiate the NA from the caudate nuclei. Thus, although our

results suggest that the NA may be relatively spared in PAE, further investigation using more sensitive methods, such as diffusion tensor imaging or higher resolution structural imaging, is needed.

PAE was also associated with volumetric reductions in both the left and right hippocampi – effects that remained significant after control for TIV. Reductions in pyramidal and granule cell number and density were observed in rats exposed prenatally to alcohol (Livy et al., 2003), which suggests that this may be the case in humans. The hippocampal formation has been shown to be particularly sensitive to prenatal alcohol exposure, showing a diverse range of negative effects and is one of the few areas of the brain which continues to exhibit these deficits as it develops into adulthood (see review, Gil-Mohapel et al., 2010). Associations of smaller hippocampi with better memory performance in typically-developing young adults (Van Petten et al., 2004) suggest a neural pruning process with age that may alter the relation of hippocampal volume in healthy controls as they grow older. Although our findings for the caudate nuclei, NA and CC are consistent with those of Archibald and associates (2001), who used a similar tracing protocol to ours, fetal alcohol-related differences in hippocampal volumes in their study did not survive after controlling for TIV. This disparity in results may be due to the wide range of ages included in that study (7-24 yr) compared to the more limited range in ours (9-11 yr). The observed association of smaller hippocampal volume with better learning and memory performance in the FAS/PFAS group in the current study was not expected at this age and is difficult to interpret.

PAE was associated with smaller CC after adjustment for TIV and postnatal lead exposure. However, regression analyses showed that the effects of smoking and alcohol on CC volume were confounded in this sample, and both fell slightly short of conventional levels of statistical significance when included in the same analysis. The effects of maternal smoking on the child's brain are not well understood, but decreases in CC size and changes in microstructure of other frontal WM structures have been linked with maternal smoking during pregnancy (Jacobsen et al., 2007; Jacobson et al., 2008; Paus et al., 2008). Given that the PAE and maternal smoking effects were similar in magnitude when adjusted for each other in the regression analysis, neither effect appears to be attributable to the effect of the other exposure, and a larger sample would likely have shown their effects to be independent.

The hypothesis that volumetric reductions in the caudate nuclei and hippocampi would mediate effects of PAE on cognitive performance was not supported. Volumetric reductions in the caudate nuclei did not relate to the cognitive outcomes; and, contrary to expectation, the hippocampal volumes in the FAS/PFAS group were inversely related to learning and memory performance.

In this study, larger CC area was associated with higher IQ scores and, amongst children in the FAS/PFAS group, with better learning and memory performance. Mediation analysis indicated that the effect of PAE on IQ was partially mediated by reductions in CC size. Our findings are consistent with several studies that have suggested that WM is more susceptible to the teratogenic effects of PAE than cortical GM (Archibald et al., 2001; Lebel et al., 2008; Spottiswoode et al., 2011). It is of interest that microstructural changes in two regions of the CC have also been found to mediate effects of PAE on IQ (Fan et al., 2016).

Morphology of the CC has been shown to have functional effects, with a thinner midline being associated with poorer motor activity and thicker than average midline being associated with poorer executive function (Bookstein et al., 2002). The CC is particularly important for functional outcomes requiring interhemispheric transfer of information, and it has been shown that even moderate exposure to alcohol may influence this process (Roebuck et al., 2002; Dodge et al., 2009). Since cognitive function assessed on IQ tests depends on a broad range of cognitive domains (Wechsler, 2003; Willoughby et al., 2008) utilizing a range of GM areas that communicate via axons in the CC, poorer interhemispheric transfer could contribute to lower IQ (Dodge et al., 2009). Smaller CC size may be due to either poorer myelination or fewer axons in the CC, resulting in slower signal processing or communication with greater numbers of GM cell bodies, respectively. CC volume reductions may also be due to changes to CC projection neurons, as reductions in size, number and location of these cells were observed in rats prenatally exposed to alcohol (Livy and Elberger, 2008). However, as no one animal model exhibits all the features of FASD (Cudd, 2005), further investigation into the underlying physiological mechanisms in humans is needed to better understand the links between CC and behavioural outcomes. As such, it is not surprising that the most heavily exposed children, those in the FAS/PFAS group, had the smallest CC areas and the lowest IQ scores.

One potential limitation of this study that is common to other longitudinal PAE studies is that it relies on the mother's report to assess alcohol consumption. However, we have previously validated the timeline follow-back interview used in this study in relation to levels of fatty acid ethyl ester metabolites in meconium samples obtained from newborns in this cohort (Bearer et al., 2003). In addition, this timeline follow-back interview has been shown to predict a broad range of behavioural (Jacobson et al., 2002, 2008; Lewis et al., 2015; Lindinger et al., 2016; Fortin et al., 2017) and neuroimaging outcomes (DeGuio et al., 2014; du Plessis et al., 2014; Meintjes et al., 2014; Jacobson et al., 2017). A second limitation relates to the difficulty assessing effects of timing of exposure in a human study. Animal models have demonstrated that timing of exposure is a major determinant of specific long-term neurobehavioral outcomes, since different brain regions are susceptible to PAE during unique sensitive periods in their development (Livy et al., 2003; Sadrian et al., 2014; Valenzuela et al., 2012). Unfortunately, multicollinearity between maternal alcohol use at conception and later in pregnancy in this cohort, as in most human samples, was too high to discriminate effects attributable to timing of exposure. Polysubstance abuse is another common limitation in PAE studies. The current study assessed potential confounding by prenatal cocaine, marijuana and smoking exposure in order to address this problem; none of these exposures were related to the outcomes examined. Strengths of this study include the use of a sample size that was large relative to other manual tracing studies, limited age range, a population that is geographically and culturally homogenous albeit diverse in terms of genetic ancestry, and the availability of continuous measures of prospectively collected alcohol consumption.

In conclusion, this study, which used manual tracing, confirms previous reports linking PAE to decreases in subcortical volumes in the caudate nucleus and hippocampus after adjustment for TIV and also showed that PAE is associated with a disproportionate reduction in CC size. This is the first study to provide evidence that fetal alcohol-related reductions in CC partially mediate effects of PAE on IQ. This finding adds to the growing body of evidence suggesting that WM may be particularly vulnerable to the teratogenic effects of PAE on development of the fetal brain.

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Chapter 3: Validity of automated FreeSurfer subcortical and corpus callosal segmentation in pre-adolescent children in studies of fetal alcohol spectrum disorders

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Abstract

In recent years a number of semi-automated and automated segmentation tools and brain atlases have been developed to facilitate morphometric analyses of large MRI datasets. The programmes are much faster than manual tracing and will produce identical results every time. However, manual tracing remains the "gold standard" of morphometric analysis, with automated results on both healthy and pathological adult brains varying across studies. Structures like the caudate nucleus show less variance between automated tracing methods, while others, like the hippocampus, show much wider variance. It remains uncertain to what extent smaller brain volumes and potential changes in grey/white matter contrasts in paediatric brains impact the performance of automated methods nor how pathology influences it. This study aimed to compare automated segmentation by FreeSurfer to manual tracing and determine its ability to detect alcohol-related volume changes in subcortical regions and the corpus callosum (CC) in children. As automated programmes are constantly being updated, both FreeSurfer version 5.1 and 6.0 were compared to manual tracing to determine whether newer versions are becoming more accurate. High-resolution T1-weighted images were acquired, using a sequence optimized for morphometric neuroanatomical analysis, on a Siemens 3T Allegra MRI scanner from 71 right-handed, 9- to 11-year-old children (27 fetal alcohol syndrome (FAS) and partial FAS (PFAS), 25 non-syndromal heavily exposed (HE) and 19 non-exposed controls) from a high-risk community in Cape Town, South Africa. Frequency of maternal drinking was ascertained prospectively during pregnancy using timeline follow-back interviews. Automated segmentation and

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manual tracing were compared statistically for linear association, consistency and absolute agreement and Cronbach's alphas were calculated. Our results support findings from other studies showing good agreement, both for the sample as a whole and within each FASD diagnostic group, between manual and automated segmentation of the caudate nucleus, which is relatively easy to segment. In contrast, since the NA and hippocampus are more difficult to delineate, consistency was moderate to good but absolute agreement was poor, while for the CC, both consistency and absolute agreement were poor. After controlling for confounders and adjustment for TIV, it is impressive that the more recent FreeSurfer version 6.0 provided evidence of alcohol-related volumetric brain reductions in the right hippocampus comparable to manual segmentation. The relative success of automated FreeSurfer segmentation version 6.0 in this sample of 9-11-year-old children, compared to the healthy, Western adults that were used to develop FreeSurfer, suggests that it can be applied with relative confidence to a geographically different, clinical cohort at this age.

3.1 Introduction

As part of ongoing studies of fetal alcohol spectrum disorders (FASD), we recently reported that heavy prenatal alcohol exposure (PAE) is associated with volume reductions in 9- to 11-year old children in the corpus callosum and bilaterally in the caudate nucleus and hippocampus using manual tracing (Biffen et al., 2018). Although manual segmentation by hand tracing remains the gold standard for measuring structure volumes (Barnes et al., 2008; Boccardi et al., 2011; Dewey et al., 2010; Fischl et al., 2002; Morey et al., 2009), it is time consuming and considered impractical for large datasets. Hand tracing of a single complex subcortical structure, such as the hippocampus can take from 1-2 hours (Akudjedu et al., 2018; Dewey et al., 2010; Morey et al., 2009), and an entire brain takes 1week or more to trace (Fischl et al., 2002). Moreover, it has been shown that even when using a single tracer, drift occurs over time due to fatigue (Zou et al., 2004).

In recent years a number of semi-automated and automated segmentation tools and brain atlases have been developed (see Helms, 2016 for a review) to facilitate morphometric analyses of large datasets. Automated segmentation of a single brain performed with the same software on the same hardware and operating system would produce identical results every time (Gronenschild et al., 2012). However, when compared to manual segmentation, performance of automated methods in both healthy (Cherbuin et al., 2009; Morey et al.,

2009; Patenaude et al., 2011) and pathological (Akhondi-Asl et al., 2011; Dewey et al., 2010; Doring et al., 2010; Guenette et al., 2018; Lehmann et al., 2010; Nugent et al., 2013; Pardoe et al., 2009; Pipitone et al., 2014; Sánchez-Benavides et al., 2010; Shen et al., 2010; Tae et al., 2008) adult populations have varied. A recent study in which a typical neuropsychiatric MRI dataset was segmented using three automated segmentation methods (FreeSurfer, FSL-FIRST and volBrain) in comparison to manual tracing demonstrated good correlation (0.77 to 0.87) on an "easy-to-segment" structure, such as the caudate nucleus, while a more difficult structure, such as the hippocampus, achieved moderate correlation for volume (0.35 to 0.62) but poor absolute agreement (ICC 0.07 to 0.10) (Akudjedu et al., 2018). While absolute volume differences may reflect differences in segmentation protocol, correlation analyses provide a measure of consistency between methods. Taken together, findings from previous studies suggest that performance of automated methods depends on the segmented structure as well as the protocol used and that discrepancy is greater in atrophic brains (Sánchez-Benavides et al., 2010) and smaller brain regions (Guenette et al., 2018; Lehmann et al., 2010; Schoemaker et al., 2016).

To date, it remains uncertain to what extent smaller brain volumes and potential changes in grey/white matter contrasts in paediatric brains impact the performance of automated methods. We found only one study in children (age 6-11 yr) which examined accuracy of automated subcortical segmentation (Schoemaker et al., 2016). Hippocampal and amygdala volumes using FreeSurfer and FSL-FIRST, respectively, showed moderate (0.61 to 0.77) and weak (0.31 to 0.59) correlations with those from manual segmentation, and all intraclass correlations (ICC) except left hippocampus volume from FreeSurfer failed to reach 0.70 (Schoemaker et al., 2016). Performance may be worse in paediatric conditions, such as fetal alcohol syndrome (FAS), that are characterised by small head circumference and reduced total brain volume (Hoyme et al., 2005).

Since the aim of many research studies is to examine brain changes associated with pathology, the ability and accuracy of automated techniques in distinguishing individuals from different clinical groups may be more important than absolute agreement. While FreeSurfer performs reasonably well in this regard in patients with Alzheimer's Disease, producing similar volume reductions (Lehmann et al., 2010; Shen et al., 2010) and hippocampal atrophy rates (Mulder et al., 2014) as manual segmentation, automated methods have been less successful in distinguishing between groups or identifying associations with

behavioural/clinical outcomes in other pathologies. For example, in patients with HIV, the association of caudal, putamen, amygdala, and hippocampal volumes with clinical measures of disease progression differed for outputs generated by FreeSurfer, IBASPM (Individual Brain Atlases using Statistical Parametric Mapping) and auto-assisted manual tracings (Dewey et al., 2010). Depression-related hippocampal volume reductions were detected with FreeSurfer but not FSL-FIRST (FMRIB Integrated Registration and Segmentation Tool, Oxford University, Oxford, UK) (Morey et al., 2009), and in former National Football League (NFL) players with neurobehavioral symptoms, automated FreeSurfer segmentation identified group differences relative to age-matched controls in 4 of 11 regions, compared to 8 of 11 with manual correction, as well as different regions showing associations with neurobehavioral factors (Guenette et al., 2018). Due to an absence of subgroups, Schoemaker et al. (2016) could not examine this question in their study on segmentation accuracy in children. Notably, in our research on 5-year-old children with HIV, automated segmentation with FreeSurfer yielded no volumetric differences compared to uninfected controls in basal ganglia, other than reductions in left globus pallidus and a tendency to larger corpus callosums. In contrast, manual segmentation demonstrated HIV-related volume *increases* in the left globus pallidus and bilaterally in the nucleus accumbens and putamen as well as corpus callosum *reductions* (Randall et al., 2017).

In the present work, we examine whether automated segmentation using FreeSurfer alters our ability compared to manual segmentation to detect alcohol-related volume changes in subcortical regions and the corpus callosum (CC) in children. To date, the impact of segmentation method when assessing effects of PAE has only been investigated in the cerebellum (Cardenas et al., 2014) using the Cerebellar Analysis Toolkit (CATK), which was able to detect changes related to PAE in 20 children and adolescents (aged 10 to 18 yr) similar to those found using manual tracing. Despite a known tendency to overestimate subcortical volumes (Cherbuin et al., 2009; Dewey et al., 2010; Doring et al., 2010; Schoemaker et al., 2016; Tae et al., 2008), we chose to use FreeSurfer as it has been shown to perform better than other automated methods on some of the structures we were interested in (Dewey et al., 2010; Morey et al., 2009; Schoemaker et al., 2016) with greater power to detect group differences (Morey et al., 2009). Since FreeSurfer was updated during our study, we present comparisons to outputs from manual tracing for two versions to examine progress towards gold standard manual segmentation.

3.2 Methods

3.2.1 Participants

Participants consisted of children born to Cape Coloured (mixed ancestry) pregnant women recruited from 1998-2002 from an antenatal clinic in Cape Town, South Africa (Jacobson et al., 2008), where prevalence of alcohol abuse is unusually high and fetal alcohol syndrome (FAS) is among the highest in the world (May et al., 2013). Pregnant women were screened and recruited at enrolment and interviewed twice during pregnancy and once within 1 month postpartum (to capture 3rd trimester drinking) regarding alcohol consumption using the timeline follow-back approach (Jacobson et al., 2002b). The volume of alcohol consumed was converted to ounces of absolute alcohol (AA; 1 oz AA is the equivalent of 2 standard drinks). At time of recruitment binge drinking was still 5 or more drinks per occasion. Any woman who reported alcohol consumption of at least 1 oz AA/day or at least 2 instances of binge drinking within their first trimester were invited to participate. Pregnant women from the same clinic were invited to participate as controls if they reported abstaining or minimal alcohol consumption and no binge drinking. Participants were also interviewed each time about their illicit drugs (cocaine and/or marijuana) used/day and number of cigarettes smoked/day during pregnancy.

Exclusion criteria for participation were age <18 years or chronic medical problems, such as diabetes, epilepsy or cardiac problems. Infants from multiple births were excluded, as well as infants presenting with major chromosomal anomalies, seizures and neural tube defects.

The children were independently examined by two expert dysmorphologists (H.E. Hoyme, M.D., and L.K. Robinson, M.D.) at a FASD diagnostic clinic we organized in 2005. Children were examined for growth and FAS-related dysmorphic features using a standard protocol (Hoyme et al., 2005) based on the Revised Institute of Medicine criteria. The determination of which children met the criteria for diagnosis with FAS or partial FAS (PFAS) was made during case conferences with the dysmorphologists and SWJ, JLJ, CDM. If children did not fall into either of these diagnostic groups they were placed, based on maternal alcohol consumption, into either the non-syndromal heavily exposed (HE) group or the control group. Diagnoses of the children were confirmed by examinations in follow-up diagnostic clinics in

which HEH was again the lead dysmorphologist in 2009, 2013 and 2016. For purposes of the analyses, data from the two syndromal groups, FAS and PFAS, were combined.

3.2.2 Magnetic Resonance Image (MRI) Acquisition

81 right-handed children from the Cape Town Longitudinal Cohort were scanned at the Cape Universities Brain Imaging Centre (CUBIC) using a 3T Siemens Allegra MRI (Siemens Medical Systems, Erlangen, Germany) when they were 9-11 years old (Meintjes et al., 2014). High-resolution T1-weighted images were obtained using a volumetric navigated (Tisdall et al., 2012) multi-echo magnetization prepared rapid gradient echo (MEMPRAGE) sequence. This sequence was optimized for analysis with FreeSurfer (van der Kouwe et al., 2008). Imaging parameters were: FOV 256×256 mm^2, 128 sagittal slices, TR 2530 ms, TE 1.53/3.21/4.89/6.57 ms, TI 1100 ms, flip angle 7°, voxel size 1.3×1.0×1.3 mm^3, acquisition time 8:07 minutes. Real-time motion tracking and correction by the volumetric navigator reduced artefacts resulting from motion.

Ethics approval was obtained from the human research ethics committees of the University of Cape Town Faculty of Health Sciences and Wayne State University. Written informed consent was obtained from the mothers/guardians and oral assent from the children.

3.2.3 Segmentation Protocol

The volumes of the CC, nucleus accumbens (NA), caudate nuclei and hippocampi were measured via manual tracing and automated segmentation. A blinded neuroanatomical researcher (SB) traced all of these structures manually on MR images with Mulitracer (Woods, 2003) software on a Wacom tablet laptop (Lenovo ThinkPad X200) using a previously described protocol (Chapter 2; Biffen et al., 2018). Briefly, hippocampal tracings only included grey matter and neither the alveous (a white matter sheath surrounding the anterior hippocampus) nor the fornices (white matter projections from the hippocampus). The caudate nucleus and NA were traced together using the lateral ventricle as the medial border and surrounding white matter (WM) as the lateral border. These two structures were separated by drawing a straight line between the inferior border of the lateral ventricle and the border of the internal capsule. MultiTracer "frust" volumes were computed for each structure. SB also ran the segmentation and parcellation protocol using FreeSurfer (version

6.0.0). Since these images had previously been analysed with an older FreeSurfer version (version 5.1.0), those data were also included in the analyses for comparison.

Ten participants were excluded - 3 due to pathology unrelated to the research question (*n*=2 HE; *n*=1 control) and 7 due to compromised image quality (*n*=1 FAS; *n*=2 HE; *n*=4 control). Pathological exclusion was due to two participants having occlusion of their lateral ventricles, which meant that the delineation separating the NA and caudate nucleus could not be performed and one participant having inclusions superior to the lateral ventricle that could potentially have indicated a conflicting diagnosis.

3.2.4 Statistical Analyses

Statistical analyses were performed in SPSS statistical package version 25 (SPSS Inc, Chicago, IL). To assess intra- and inter-rater reliabilities of manually traced ROIs, images of 10 randomly selected participants were retraced by the primary researcher (SB) and another experienced neuroanatomist (SR), using the same protocol. Pearson correlation was performed to examine the strength of association between volume measurements, intraclass correlation (two-way mixed-effects model) to test consistency (correspondence) and absolute agreement (presence of systematic differences), and Cronbach's alpha (α) to determine internal consistency. These same tests were used to separately compare ROI volumes from manual tracing with automated segmentations from each version of FreeSurfer, and volumes from the two versions of FreeSurfer with each other. For each region, volumes from all three segmentations were compared using ANOVA in the sample as a whole and within each FASD diagnostic group. The latter served to examine whether segmentation performance of automated methods would be more optimal in healthy children than those with PAE. For CC and total intracranial volume (TIV), only volumes from the two FreeSurfer versions were compared using pairwise *t*-tests, as manual tracing measures CC area and TIV had not been traced manually.

To compare the accuracy of the different segmentation methods in distinguishing participants based on diagnosis, we examined volumetric differences between the diagnostic groups separately for each segmentation method using ANOVA. Potential control variables included child sex and age at scan (yr), socioeconomic status (SES; Hollingshead, 2011), maternal smoking during pregnancy (cigarettes/day), and postnatal lead exposure (ug/dl) obtained

from a blood sample at 5 years. ANCOVA was used to examine whether volumetric group differences remained significant after controlling for confounders even weakly related to the outcome at $p<0.10$. An additional ANCOVA was run to examine whether the region in question was disproportionately smaller when PAE-related whole brain volume (TIV) reductions were considered.

Pearson correlation was used to examine the association between a continuous measure of alcohol exposure (AA/day) and the structure volumes obtained from each segmentation method. Multiple regression was used to control for control variables weakly related to the outcome (at $p<0.10$), and TIV was included in an additional step to determine whether regions were disproportionately affected compared to whole brain volume reductions related to PAE.

In group comparisons and correlation analyses involving volumes measured by a specific FreeSurfer version, TIVs from that version of FreeSurfer were used. For analyses of data from manual tracing, TIVs from the latest FreeSurfer version (v. 6.0) were used.

3.3 Results

Sample characteristics for the 71 children and their mothers are summarized in Table 3.1. Children in the FAS/PFAS and control groups were slightly younger than those in the HE group. As expected, those in the FAS/PFAS group had lower IQs, and TIVs were smaller for the syndromal FAS/PFAS group than the non-syndromal HE and control groups. Groups did not differ by sex or childhood lead exposure.

Mothers in the FAS/PFAS group smoked more cigarettes than those in the control group. By design, in all measures of alcohol consumption, mothers of children in the FAS/PFAS and HE groups consumed more alcohol than mothers of control children, with the FAS/PFAS mothers consuming more than the HE mothers in all measures except AA/drinking day (oz) across pregnancy. The key difference between mothers of the FAS/PFAS and HE groups is that the FAS/PFAS mothers binge drink more frequently – drinking twice as often per week. All but one mother in the control group abstained from drinking during pregnancy; that mother consumed only 2 drinks/occasion on 2-3 days during pregnancy. Marijuana and cocaine use were rare.

Table 3.1: Sample characteristics (N=71)

		Control		Exposed HE		Exposed FAS/PFAS		TOTAL		F or X^2
		(*n*=19)		(*n*=25)		(*n*=27)		(*N*=71)		
Child:										
Sex: Male	*n*(%)	9	(47%)	16	(64%)	14	(52%)	39	(55%)	1.37
Age (yr) at scan[a]	Mean (SD)	10.6	(0.5)	11.0	(0.7)	10.5	(0.6)	10.7	(0.7)	4.05**
WISC-IV IQ[b]	Mean (SD)	73	(10.7)	76	(11.1)	64	(11.0)	71	(12.1)	8.73***
TIV (x10^6 mm^3)[c]	Mean (SD)	1.4	(0.1)	1.5	(0.1)	1.3	(0.1)	1.4	(0.1)	6.76**
Lead (ug/dl)	Mean (SD)	9.2	(3.5)	9.4	(3.3)	11.6	(5.9)	10.2	(4.6)	2.16
Maternal:										
Cigarettes/day[d, #]	Median(IQR)	0.0	(5.0)	5.3	(8.3)	7.5	(5.0)	5.0	(10.0)	2.38*
Marijuana (yes)	*n* (%)	0	(0%)	2	(8%)	3	(11%)	5	(7%)	2.16
Cocaine (yes)	*n* (%)	0	(0%)	0	(0%)	1	(3.7%)	1	(1%)	1.65
Alcohol at conception										
AA/day (oz)[e]	Median(IQR)	0.0	(0.0)	0.6	(1.0)	1.3	(1.3)	0.6	(1.3)	35.67****
AA/drinking day (oz)[f]	Mean (SD)	0.06	(0.3)	2.8	(2.2)	4.6	(2.4)	2.8	(2.7)	28.50****
Drinking days/wk[g]	Mean (SD)	0.0	(0.1)	1.4	(1.1)	2.7	(1.6)	1.5	(1.6)	26.90****
Alcohol across pregnancy										
AA/day (oz)[h]	Median(IQR)	0.0	(0.0)	0.2	(0.7)	1.0	(0.8)	0.3	(1.0)	31.61****
AA/drinking day (oz)[i]	Mean(SD)	0.06	(0.3)	3.4	(2.4)	4.2	(1.8)	2.8	(2.5)	31.04****
Drinking days/wk[j]	Mean (SD)	0.0	(0.0)	1.0	(0.8)	2.0	(1.3)	1.1	(1.2)	26.47****

For skewed data medians and interquartile ranges (IQR) were used. HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS; WISC-IV Wechsler Intelligence Scales for Children-Fourth Edition; TIV total intracranial volume as measured by FreeSurfer v. 6.0.

[#]Winsorized data used (PFAS/FAS *n*=0, HE *n*=0, Control *n*=1)

[a] HE > FAS/PFAS (*p*=0.007), Control (*p*=0.06)

[b] FAS/PFAS < HE (*p*<0.001), Control (*p*=0.006)

[c] FAS/PFAS < HE (*p*=0.001), Control (*p*=0.035)

[d] FAS/PFAS > Control (*p*=0.035)

[e] Control < FAS/PFAS, HE (both *p*<0.001); HE < FAS/PFAS (*p*<0.001)

[f] Control < FAS/PFAS, HE (both *p*<0.001); HE < FAS/PFAS (*p*=0.002)

[g] Control < FAS/PFAS, HE (both *p*<0.001); HE < FAS/PFAS (*p*<0.001)

[h] Control < FAS/PFAS, HE (both *p*<0.001); HE < FAS/PFAS (*p*<0.001)

[i] Control < FAS/PFAS, HE (both *p*<0.001)

[j] Control < FAS/PFAS, HE (both *p*≤0.001); HE < FAS/PFAS (*p*<0.001)

*$p\leq0.1$, ** $p\leq0.05$, ***$p\leq0.01$, ****$p\leq0.001$

In all brain regions, except left NA, intra-rater reliabilities for all tests were greater than 0.93 (Table 3.2), indicating that the same rater can repeatedly trace the same structures with good agreement. In left NA, values on the different tests ranged from 0.74 (Pearson correlation) to 0.84 (ICC absolute agreement). Inter-rater reliabilities assessed on these measures were 0.89 or higher in all regions except the left NA, where it ranged from 0.71 (Pearson correlation) to 0.83 (all other measures).

Table 3.2: Intra- and inter-rater reliability of manually traced subcortical and corpus callosum volumes for ten randomly selected participants.

	Correlations	ICC (Consistency)	ICC (Absolute Agreement)	Cronbach's α
ROI	*r*(*p*)	*r*(*p*)	*r*(*p*)	
A. INTRA-RATER RELIABILITY				
Grey matter ROIs				
L Caudate	0.994 (<0.001)	0.997 (<0.001)	0.994 (<0.001)	0.997
R Caudate	0.991 (<0.001)	0.995 (<0.001)	0.995 (<0.001)	0.995
L NA	0.742 (0.014)	0.832 (0.007)	0.838 (0.007)	0.832
R NA	0.945 (<0.001)	0.972 (<0.001)	0.968 (<0.001)	0.972
L Hippocampus	0.978 (<0.001)	0.989 (<0.001)	0.967 (<0.001)	0.989
R Hippocampus	0.933 (<0.001)	0.965 (<0.001)	0.967 (<0.001)	0.965
White matter ROI				
CC	0.990 (<0.001)	0.995 (<0.001)	0.988 (<0.001)	0.995
B. INTER-RATER RELIABILITY				
Grey matter ROIs				
L Caudate	0.983 (<0.001)	0.989 (<0.001)	0.990 (<0.001)	0.989
R Caudate	0.990 (<0.001)	0.995 (<0.001)	0.995 (<0.001)	0.995
L NA	0.710 (0.021)	0.826 (0.008)	0.830 (0.008)	0.826
R NA	0.898 (<0.001)	0.946 (<0.001)	0.900 (<0.001)	0.946
L Hippocampus	0.956 (<0.001)	0.976 (<0.001)	0.950 (<0.001)	0.976
R Hippocampus	0.889 (0.001)	0.940 (<0.001)	0.899 (<0.001)	0.940
White matter ROI				
CC	0.926 (<0.001)	0.956 (<0.001)	0.960 (<0.001)	0.956

NA nucleus accumbens; CC corpus callosum

Caudal volumes from manual tracing showed very strong correlations (≥ 0.86), excellent consistency (> 0.92) and absolute agreement (> 0.91) with volumes obtained using both FreeSurfer versions 5.1 and 6.0 (Tables 3.3A and 3.3B). While correlation and consistency of hippocampal volumes from both FreeSurfer versions compared to manual tracing were moderate to strong (0.60 to 0.82), absolute agreement was poor (0.11 to 0.15). By contrast, correlations (0.35 to 0.64), consistency (0.50 to 0.77), and absolute agreement (0.15 to 0.40) were poor to moderate for NA volumes, with FreeSurfer version 6.0 consistently yielding better results in this region. Although CC volumes showed strong correlations ($r > 0.83$), consistency and agreement were poor. The latter is likely due to the fact that FreeSurfer measures a volume, whereas we manually traced CC area. Caudal and CC volumes from the two different versions of FreeSurfer (Table 3.3C) yielded values on all measures >0.92. Measures for hippocampi were all greater than 0.82, and for NA all measures, except absolute agreement for right NA, were greater than 0.70.

Caudal volumes did not differ between the different segmentation methods (Table 3.4), but manually traced NA and hippocampal volumes were smaller than those obtained using either version of FreeSurfer. Whereas NA and left hippocampal volumes from FreeSurfer v.6.0 were smaller than those from FreeSurfer v.5.1, right hippocampal volume, CC and TIV were larger. The findings within each FASD diagnostic group were similar (Fig. 3.1).

Table 3.3: Correlation, consistency and absolute agreement of ROI volumes from (A) manual tracing and automated segmentation with FreeSurfer v.5.1, (B) manual tracing and FreeSurfer v.6.0, and (C) the two different FreeSurfer versions (N=71).

ROI	Correlations *r*(*p*)	ICC (Consistency) *r*(*p*)	ICC (Absolute Agreement) *r*(*p*)	Cronbach's α
A. MANUAL TRACING vs FreeSurfer v. 5.1				
Grey matter ROIs				
L Caudate	0.860 (<0.001)	0.925 (<0.001)	0.924 (<0.001)	0.925
R Caudate	0.858 (<0.001)	0.923 (<0.001)	0.919 (<0.001)	0.923
L NA	0.350 (0.003)	0.503 (0.002)	0.233 (0.002)	0.503
R NA	0.430 (<0.001)	0.572 (<0.001)	0.150 (<0.001)	0.572
L Hippocampus	0.601 (<0.001)	0.751 (<0.001)	0.113 (<0.001)	0.751
R Hippocampus	0.680 (<0.001)	0.809 (<0.001)	0.144 (<0.001)	0.809
White matter ROI				
CC	0.847 (<0.001)	0.446 (0.007)	0.021 (0.007)	0.446
B. MANUAL TRACING vs FreeSurfer v. 6.0				
Grey matter ROIs				
L Caudate	0.893 (<0.001)	0.942 (<0.001)	0.930 (<0.001)	0.942
R Caudate	0.910 (<0.001)	0.952 (<0.001)	0.953 (<0.001)	0.952
L NA	0.492 (<0.001)	0.659 (<0.001)	0.364 (<0.001)	0.659
R NA	0.636 (<0.001)	0.773 (<0.001)	0.397 (<0.001)	0.773
L Hippocampus	0.665 (<0.001)	0.799 (<0.001)	0.153 (<0.001)	0.799
R Hippocampus	0.701 (<0.001)	0.823 (<0.001)	0.139 (<0.001)	0.823
White matter ROI				
CC	0.837 (<0.001)	0.418 (0.013)	0.019 (0.013)	0.418
C. FreeSurfer v. 5.1 vs FreeSurfer v. 6.0				
TIV	0.692 (<0.001)	0.817 (<0.001)	0.773 (<0.001)	0.817
Grey matter ROIs				
L Caudate	0.926 (<0.001)	0.961 (<0.001)	0.937 (<0.001)	0.961
R Caudate	0.953 (<0.001)	0.976 (<0.001)	0.971 (<0.001)	0.976
L NA	0.709 (0.003)	0.802 (<0.001)	0.751 (<0.001)	0.802
R NA	0.717 (<0.001)	0.820 (<0.001)	0.565 (<0.001)	0.820
L Hippocampus	0.828 (<0.001)	0.905 (<0.001)	0.853 (<0.001)	0.905
R Hippocampus	0.848 (<0.001)	0.918 (<0.001)	0.907 (<0.001)	0.918
White matter ROI				
CC	0.952 (<0.001)	0.974 (<0.001)	0.937 (<0.001)	0.974

NA nucleus accumbens; CC corpus callosum, TIV total intracranial volume

Table 3.4: Comparison of ROI volumes (mm^3) using different segmentation methods.

ROI	Manual (*N*=71)	FreeSurfer v. 5.1 (*N*=71)	FreeSurfer v. 6.0 (*N*=71)	*p*
TIV ($x10^6 mm^3$)	-	1.326 (0.014)	1.391 (0.015)	**<0.001**♦
Grey matter ROIs				
L Caudate	3748 (567)	3798 (560)	3623 (529)	0.156
R Caudate	3783 (584)	3865 (548)	3787 (558)	0.622
L NA[a]	529 (93)	723 (125)	670 (88)	**<0.001**
R NA[b]	481 (88)	792 (130)	670 (88)	**<0.001**
L Hippocampus[c]	2349 (325)	3702 (310)	3552 (317)	**<0.001**
R Hippocampus[d]	2379 (294)	3173 (503)	3635 (322)	**<0.001**
White Matter ROI				
CC	551 (88)◊	3177 (501)	3383 (541)	**<0.001**♦

Values are mean(SD). TIV Total intracranial volume; NA nucleus accumbens; CC corpus callosum.

Bold print denotes significance at $p<0.05$

◊ Area averaged from 2 consecutive slices

♦ Paired *t*-test between volumes from different FreeSurfer versions

[a] Manual < FreeSurfer v. 5.1, FreeSurfer v. 6.0 (both $p<0.001$); FreeSurfer v. 6.0< FreeSurfer v. 5.1 ($p=0.003$)

[b] Manual < FreeSurfer v. 5.1, FreeSurfer v. 6.0 (both $p<0.001$); FreeSurfer v. 6.0< FreeSurfer v. 5.1 ($p<0.001$)

[c] Manual < FreeSurfer v. 5.1, FreeSurfer v. 6.0 (both $p<0.001$); FreeSurfer v. 6.0< FreeSurfer v. 5.1 ($p=0.005$)

[d] Manual < FreeSurfer v. 5.1, FreeSurfer v. 6.0 (both $p<0.001$); FreeSurfer v. 6.0> FreeSurfer v. 5.1 ($p<0.001$)

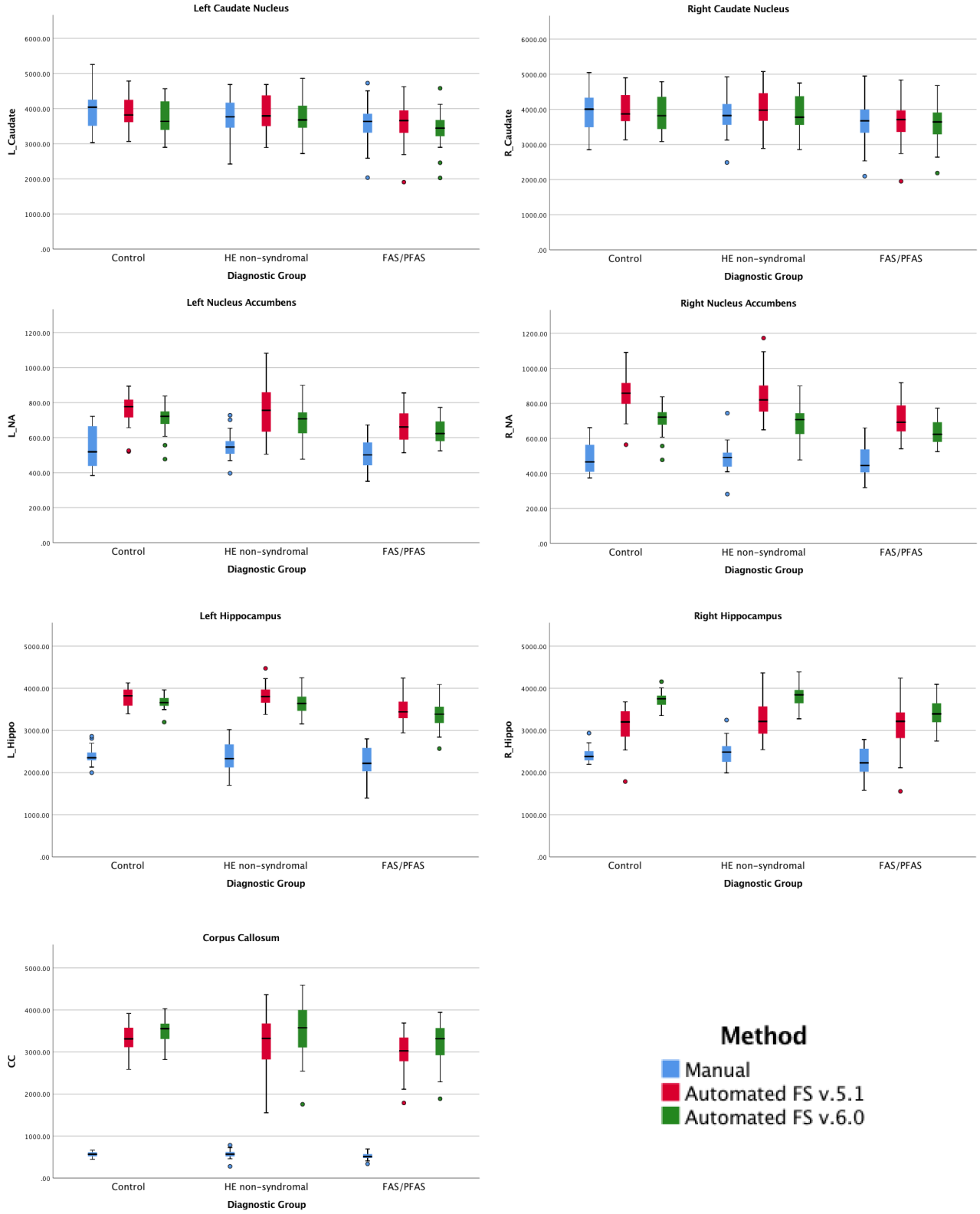

Figure 3.1: Comparison within each diagnostic group of subcortical and corpus callosum volumes (mm^3) obtained from manual and automated segmentation. Manual tracing produces a corpus callosum area (mm^2).

Lastly, we assessed sensitivity of volumes obtained from the different segmentation methods to detect effects of prenatal alcohol exposure. Whereas children with FAS/PFAS only demonstrated volume reductions in right hippocampus compared to HE and control children using manual tracing (Table 3.5A), unilateral caudal (right on version 5.1; left on version 6.0), bilateral NA and bilateral hippocampal volume reductions were evident for automatic segmentation (Tables 3.5B and 3.5C). Virtually all of the findings remained significant after control for confounders. When controlling for TIV, only right hippocampus tended to be disproportionately smaller on volumes from manual tracing and FreeSurfer v.6.0. By contrast, volumes from the older FreeSurfer v.5.1 were disproportionately smaller in children with FAS/PFAS compared to the other groups in right NA and bilateral hippocampus.

Increasing alcohol exposure was associated with decreasing caudal and hippocampal volumes using all segmentation methods – effects that remained significant after controlling for confounders and TIV (Table 3.6). Associations of NA volume with alcohol exposure were only evident in data from automated segmentation. Although higher alcohol exposure was related to smaller CC size across all methods, the findings in manually traced data did not survive after controlling for confounders.

Table 3.5: Comparison of subcortical volumes (mm^3) by diagnostic group for each segmentation method.

ROI	Control (*n*=19)	HE (*n*=25)	FAS/PFAS (*n*=27)	Total (*N*=71)	*p*	p_1	p_2
A. Manual Tracing[1]							
Grey matter ROIs							
L Caudate	3929 (563)	3785 (536)	3585 (573)	3748 (567)	0.118	0.155	0.356
R Caudate	3936 (582)	3806 (541)	3655 (617)	3783 (584)	0.272	0.294	0.406
L NA	542 (117)	549 (71)	501 (90)	529 (93)	0.143	**0.051**	0.320
R NA	490 (95)	492 (82)	464 (90)	481 (88)	0.472	0.176	0.810
L Hippocampus	2400 (224)	2394 (339)	2272 (365)	2349 (325)	0.294	0.213	0.722
R Hippocampus[a]	2432 (185)	2473 (296)	2255 (319)	2379 (294)	**0.016**	**0.002**	0.065
White matter ROI							
CC	564 (64)	569 (105)	524 (80)	551 (88)	0.128	0.452	0.896
B. Automated Segmentation FS V5.1.0[2]							
Grey matter ROIs							
L Caudate	3942 (509)	3897 (525)	3606 (591)	3798 (560)	0.072	0.090	0.193
R Caudate[b]	3977 (468)	4000 (542)	3662 (562)	3865 (548)	**0.047**	0.070	0.265
L NA[c]	753 (104)	757 (148)	670 (97)	723 (125)	**0.017**	0.108	0.212
R NA[d]	848 (118)	836 (127)	713 (101)	792 (130)	**<0.001**	**0.001**	**0.002**
L Hippocampus[e]	3780 (219)	3839 (262)	3520 (325)	3702 (310)	**<0.001**	**<0.001**	**0.013**
R Hippocampus[f]	3685 (185)	3681 (261)	3379 (333)	3567 (309)	**<0.001**	**0.004**	**0.028**
White matter ROI							
CC	3302 (320)	3265 (624)	3008 (447)	3177 (502)	0.080	0.234	0.586
C. Automated Segmentation FS V6.0.0[1]							
Grey matter ROIs							
L Caudate[g]	3777 (487)	3723 (524)	3423 (516)	3624 (529)	**0.039**	**0.037**	0.341
R Caudate	3915 (547)	3882 (527)	3611 (568)	3787 (558)	0.110	0.123	0.534
L NA[h]	704 (85)	689 (95)	630 (70)	670 (88)	**0.007**	**0.006**	0.174
R NA[i]	663 (81)	682 (113)	605 (90)	648 (101)	**0.016**	**0.010**	0.508
L Hippocampus[j]	3667 (173)	3667 (291)	3364 (336)	3552 (317)	**<0.001**	**0.003**	0.169
R Hippocampus[k]	3723 (192)	3796 (269)	3429 (332)	3637 (321)	**<0.001**	**<0.001**	0.059
White matter ROI							
CC	3492 (291)	3493 (672)	3204 (508)	3383 (541)	0.091	0.325	0.960

Values are Mean (SD). NA nucleus accumbens; CC corpus callosum; TIV total intracranial volume; HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS

Bold print denotes significance at $p \leq 0.05$

p_1 after controlling for sex of child, cigarettes/day during pregnancy, lead concentration (ug/dl), child age at scan and socioeconomic status

p_2 after controlling for TIV, sex of child, cigarettes/day during pregnancy, lead concentration (ug/dl), child age at scan and socioeconomic status

[1]FreeSurfer v. 6.0 TIV used

[2]FreeSurfer v. 5.1 TIV used

[a] FAS/PFAS<HE non-syndromal (p=0.007) and Control (p=0.039)

[b] FAS/PFAS<HE non-syndromal (p=0.025) and Control (p=0.052)

[c] FAS/PFAS<HE non-syndromal (p=0.010) and Control (p=0.023)

[d] FAS/PFAS<HE non-syndromal, Control (both p<0.001)

[e] FAS/PFAS<HE non-syndromal (p<0.001) and Control (p=0.003)

[f] FAS/PFAS<HE non-syndromal, Control (both p<0.001)

[g] FAS/PFAS<HE non-syndromal (p=0.038) and Control (p=0.024)

[h] FAS/PFAS<HE non-syndromal (p=0.012) and Control (p=0.004)

[i] FAS/PFAS<HE non-syndromal (p=0.006) and Control (p=0.052)

[j] FAS/PFAS<HE non-syndromal and Control (both $p \leq 0.001$)

[k] FAS/PFAS<HE non-syndromal and Control (both $p \leq 0.001$)

Table 3.6: Association of subcortical volumes with extent of alcohol exposure for each of the segmentation methods.

	Manual Tracing			FreeSurfer v. 5.1			FreeSurfer v. 6.0		
ROI	*r* (*p*)	β_1 (*p*)	β_2^a (*p*)	*r* (*p*)	β_1 (*p*)	β_2^b (*p*)	*r* (*p*)	β_1 (*p*)	β_2^a (*p*)
L Caudate	**-0.366**	**-0.418**	**-0.271**	**-0.335**	**-0.435**	**-0.351**	**-0.353**	**-0.467**	**-0.320**
	(0.002)	**(0.003)**	**(0.035)**	**(0.004)**	**(0.002)**	**(0.007)**	**(0.002)**	**(0.001)**	**(0.009)**
R Caudate	**-0.311**	**-0.378**	-0.249	**-0.347**	**-0.432**	**-0.347**	**-0.315**	**-0.413**	**-0.269**
	(0.008)	**(0.008)**	(0.066)	**(0.003)**	**(0.001)**	**(0.006)**	**(0.007)**	**(0.003)**	**(0.036)**
L NA	-0.142	-0.203	-0.117	**-0.272**	-0.266	-0.228	**-0.265**	**-0.336**	-0.129
	(0.239)	(0.141)	(0.399)	**(0.022)**	(0.067)	(0.122)	**(0.026)**	**(0.022)**	(0.282)
R NA	-0.085	-0.176	-0.055	**-0.369**	**-0.413**	**-0.359**	**-0.264**	**-0.366**	-0.197
	(0.481)	(0.204)	(0.681)	**(0.002)**	**(0.002)**	**(0.008)**	**(0.026)**	**(0.011)**	(0.122)
L Hippocampus	**-0.295**	**-0.391**	**-0.319**	**-0.352**	**-0.392**	**-0.259**	**-0.458**	**-0.467**	**-0.318**
	(0.012)	**(0.006)**	**(0.028)**	**(0.003)**	**(0.007)**	**(0.034)**	**(<0.001)**	**(<0.001)**	**(0.007)**
R Hippocampus	**-0.275**	**-0.394**	**-0.283**	**-0.487**	**-0.425**	**-0.337**	**-0.411**	**-0.419**	**-0.257**
	(0.020)	**(0.007)**	**(0.047)**	**(<0.001)**	**(0.001)**	**(0.005)**	**(<0.001)**	**(0.002)**	**(0.032)**
CC	**-0.335**	-0.231	-0.101	**-0.384**	**-0.292**	-0.228	**-0.400**	**-0.314**	-0.184
	(0.004)	(0.103)	(0.456)	**(0.001)**	**(0.036)**	(0.095)	**(0.001)**	**(0.023)**	(0.160)

NA nucleus accumbens; CC corpus callosum.
Bold print denotes significance at $p \leq 0.05$
r Pearson correlation coefficient
β_1 after controlling for sex of child, cigarettes/day during pregnancy, lead concentration (ug/dl), child age at scan and socioeconomic status
β_2^a after controlling for sex of child, cigarettes/day during pregnancy, lead concentration (ug/dl), child age at scan, socioeconomic status and TIV (FreeSurfer v. 6.0)
β_2^b after controlling for sex of child, cigarettes/day during pregnancy, lead concentration (ug/dl), child age at scan, socioeconomic status and TIV (FreeSurfer v. 5.1)

3.4 Discussion

Our results support findings from other studies showing good agreement, both for the sample as a whole and within each FASD diagnostic group, between manual and automated segmentation of the caudate nucleus, which is relatively easy to segment (Akudjedu et al., 2018). In contrast, since the NA and hippocampus are more difficult to delineate, consistency was moderate to good but absolute agreement was poor, while for the CC, both consistency and absolute agreement were poor. Our use of a different protocol for manual and automated

tracing of the CC limited the inferences that can be made from these reliability tests. After controlling for confounders and adjustment for TIV, it is impressive that the more recent FreeSurfer version 6.0 provided evidence of alcohol-related volumetric brain reductions in the right hippocampus comparable to manual segmentation.

Although manual tracing is considered the gold standard of volumetric measurement of structural MRIs, one of the major strengths of automated methods is that they provide the same measurements regardless of the number of iterations or researchers. Excellent to good ICC values (Koo and Li, 2016) for intra- and inter-rater reliabilities in our study confirmed that no criterion drift occurred for the single tracer over time and that another trained neuroanatomist would obtain similar results when tracing the same ROIs given the same manual tracing protocol. Commonly accepted criteria for Cronbach's α suggests that reliabilities should not be below 0.80 in basic research and should be above 0.90 for widely used scales; 0.95 and above should be the desired standard for any scale (Carmines and Zeller, 1979; Lance et al., 2006). The intra- and inter-rater reliabilities for the current study fall within this range, indicating that manual tracing is at a minimum acceptable for the left NA and reaches the desired standard for the caudate nuclei, left hippocampus and CC, even when multiple tracers are used.

In all regions except the CC, correlational analyses showed slightly stronger associations of volumes of manual segmentation with volumes from FreeSurfer v.6.0 than FreeSurfer v.5.1. ICCs for consistency and absolute agreement were also somewhat higher or similar between manual tracing and FreeSurfer v.6.0 than v.5.1. The poor absolute agreement scores (<0.40) for all regions except the caudate nuclei reflect differences in segmentation protocols, while the moderate (NA) to strong (hippocampus) to excellent (caudate) consistency scores, which tend to increase with version updates, suggest reliable systematic differences. Pearson and ICC values between the two FreeSurfer versions for NA and hippocampus that were only moderate to strong indicate that software updates have altered segmentation of these structures. Overall, improved reliability of manual tracing with FreeSurfer v.6.0 suggests that programme updates are leading to increased sensitivity and more accurate delineation of subcortical structures.

Consistency and association between manual and automated segmentation was worst for the NA, probably due to the theoretical, rather than organic, borders created to separate NA from

the caudate nucleus and putamen (Biffen et al., 2018; Looi et al., 2008; Randall et al., 2017). Figure 3.2 shows the lines used to form its superior and lateral borders. Of note in Figure 3.2 (B) is the erroneous inclusion of WM (anterior commissure) by FreeSurfer v.5.1 in the volume of the left NA, which manual tracing and FreeSurfer v.6.0 correctly excluded. Studies have also shown that smaller structures are more sensitive to variability both within and between segmentation methods (Morey et al., 2009; Schoemaker et al., 2018). The somewhat arbitrary borders and small size of the NA (Archibald et al., 2001; Biffen et al., 2018; Fischl et al., 2002) probably contribute to high variability and poor reliability in this region.

Conversely, the caudate nucleus has very clear borders and delineation is well-defined and agreed upon in the literature (Archibald et al., 2001; Fryer et al., 2012; Looi et al., 2008). Manual intra- and inter-rater reliabilities are highest in this region, as are agreement and consistency between manual and automated methods. Figure 3.2 visually confirms that the delineation of the caudate nucleus by all three segmentation methods is very similar. This confirms findings from previous studies in which the caudate exhibited the least variability between automated and manual tracing (Akudjedu et al., 2018; Dewey et al., 2010). Cronbach's α indicates that both manual tracing of this structure and FreeSurfer v. 6.0 approach the desired standard of 0.95.

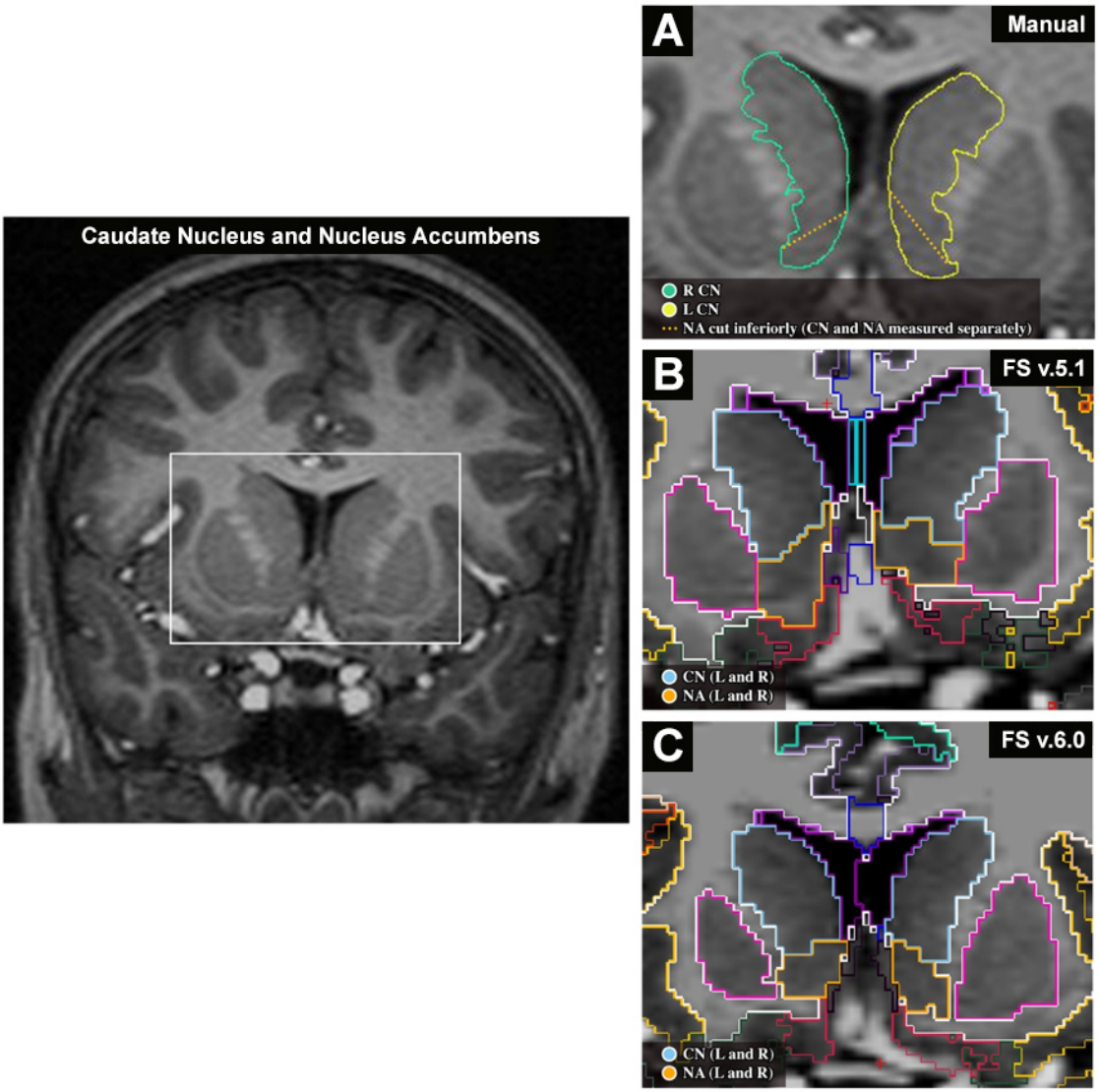

Figure 3.2: (Left) Coronal slice showing the caudate nucleus (CN), and (right) zoomed images showing segmentation of the caudate nucleus (CN) and nucleus accumbens (NA) by (A) manual tracing, (B) FreeSurfer v. 5.1 and (C) FreeSurfer v. 6.0.

Comparisons of manual and automated segmentation have often focused on the hippocampus, possibly due to the clinical implications of volume changes in this area (Akudjedu et al., 2018; Barnes et al., 2008; Dewey et al., 2010; Morey et al., 2009; Schoemaker et al., 2016). Although not reaching the same levels of reliability between FreeSurfer and manual tracing as the caudate nucleus, correlation, consistency and absolute agreement increased somewhat from version 5.1 to 6.0. Cronbach's α scores also increased in the later version suggesting that less of the variability in volume is due to error, but rather to anatomical variation. Similar to findings from other studies, hippocampal volumes were overestimated by both versions of FreeSurfer (Akudjedu et al., 2018; Dewey et al., 2010;

Schoemaker et al., 2018). Figures 3.3 and 3.4 show that this overestimation is likely due to differences in segmentation protocols. Manual tracing did not include the alveous nor the fornices. Since this white matter is difficult to differentiate from the grey matter in the coronal plane (Fig. 3.3A), the hippocampus was delineated in the sagittal plane (Fig. 3.4A and B), where the white matter is more easily visible in terms of intensity and anatomical location. In Figures 3.3B-C and 3.4C-F, it can be seen that neither version of FreeSurfer removes the alveous and that random areas of the posterior horn of the lateral ventricle (black areas) are often included. Despite this, FreeSurfer v.6.0 (Figs. 3.3C, 3.4E and 3.4F) produces a shape that is anatomically accurate and similar to that from manual tracing. The hippocampus is difficult to automatically segment due to neighbouring structures having similar intensities and high probabilities. Compared to the other structures traced manually, this structure is the one where an orthogonal plane and heuristic decision-making was relied on most heavily.

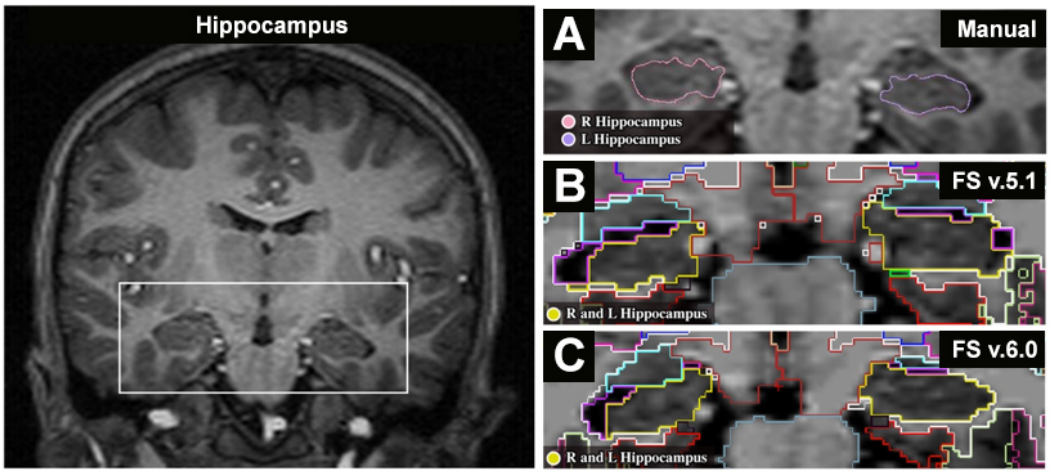

Figure 3.3: (Left) Coronal slice showing the hippocampal region, and (right) zoomed images of the boxed region showing segmentation of the left and right hippocampus by (A) manual tracing, (B) FreeSurfer v. 5.1 and (C) FreeSurfer v. 6.0.

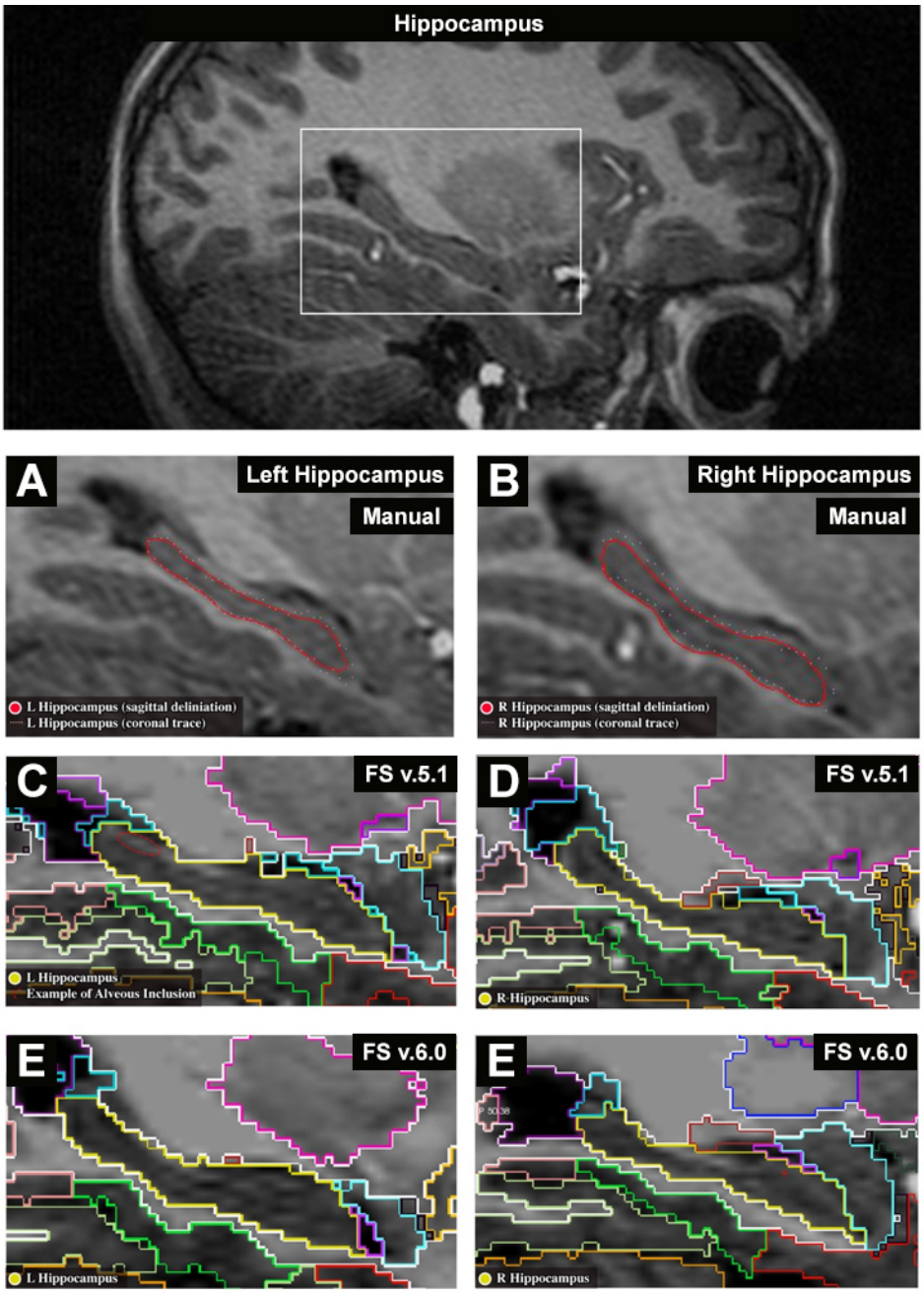

Figure 3.4: (Top) Hippocampal region on a sagittal slice, and zoomed images of the boxed region showing segmentation by (A, B) manual tracing, (C, D) FreeSurfer v.5.1, and (E, F) FreeSurfer v.6.0 for the (left column) left and (right column) right hippocampus, respectively.

The CC is probably the most difficult structure for which to assess performance based on reliabilities alone. For manual tracing we computed an average area based on the two middle-most slices, which provides a very conservative but reliable measure of the total area of white matter that crosses from one hemisphere to the other. While it does not give us any information on the quality of this white matter (for example, whether reduced volume is

related to fewer axons or decreased myelination), it does inform whether the area of interhemispheric cross-over is changed. In contrast, FreeSurfer measures the CC over a larger number of slices (often 6 or 7). The point at which the CC is separated from other white matter, such as the centrum ovale and corona radiata is difficult to determine without directional tract information, hence the conservative approach of the manual tracing protocol. Despite these differences, volumes from manual and automated segmentation were significantly associated, although both ICC consistency and absolute agreement were poor. Reliability measures were similar for comparisons of manual tracing to both versions of FreeSurfer. The midline slice of CC, seen in Figure 3.5B, shows how FreeSurfer v.5.1 includes some of the fornix into the posterior CC and the artery curving around the CC anteriorly (bright white) into the anterior and mid-anterior CC volumes. All parts of the CC were, however, still correctly identified. In contrast, FreeSurfer v. 6.0 mislabeled the anterior and posterior CC as cerebral white matter in the slice shown in Figure 3.5C.

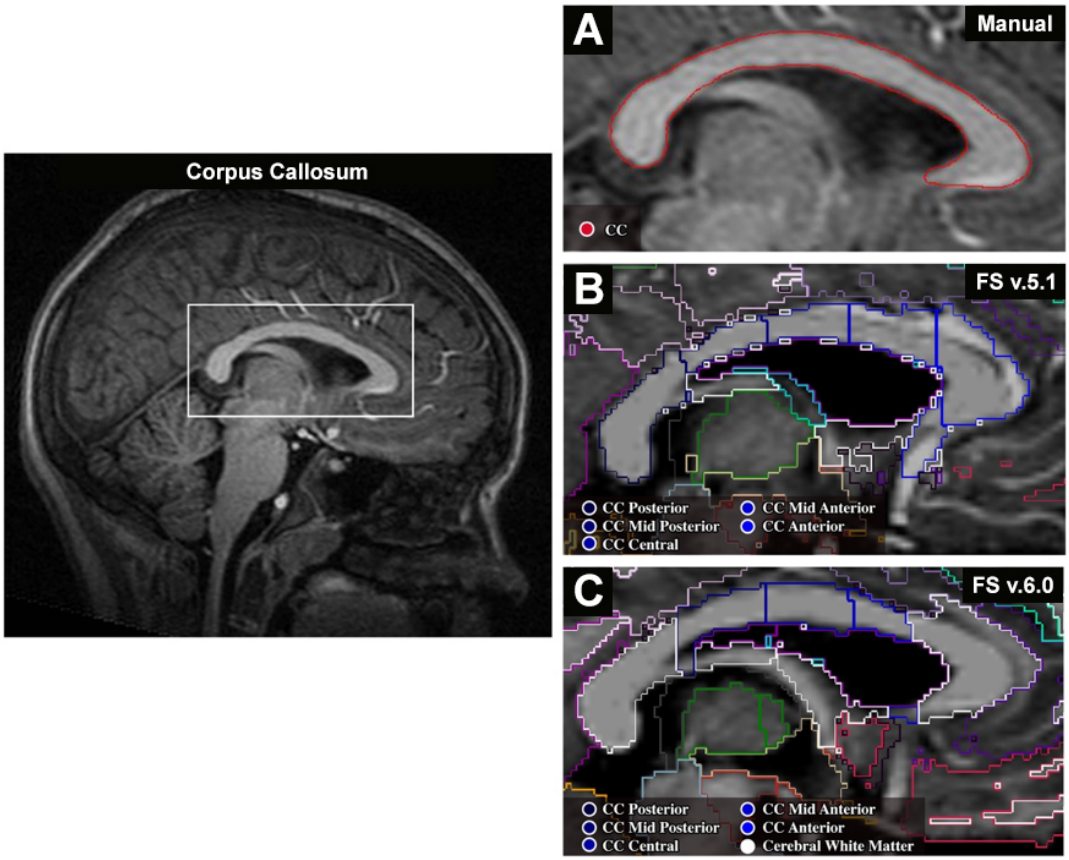

Figure 3.5: (Left) Mid-sagittal slice showing the corpus callosum, and (right) zoomed images of the boxed region showing segmentation of the CC by (A) manual tracing, (B) FreeSurfer v. 5.1 and (C) FreeSurfer v. 6.0.

Although it has been reported that discrepancy of automated segmentation is greater in atrophic brains (Sánchez-Benavides et al., 2010) , the performance of the three segmentation methods considered here was similar within each diagnostic group, except that the alcohol exposed groups displayed greater variability in some regions.

In the present work, automated segmentation identified more regions with alcohol related volume reductions than manual tracing. Differences between segmentation methods in distinguishing between groups and identifying regions related to clinical measures or behavioural outcomes have been reported in HIV (Dewey et al., 2010), depression (Morey et al., 2009), and NFL players (Guenette et al., 2018). It is possible that automated segmentation improves the power to detect group differences due to reduced variability in the data, although that is not evident in the data, except possibly in the left hippocampus (Fig. 3.1). Notably, regions from FreeSurfer v.6.0 showing alcohol-related volume reductions that did not also show volume reductions with manual tracing, did not survive when controlling for TIV, suggesting that these reductions may be a consequence of overall reduced brain size, rather than regions specifically vulnerable to alcohol. It is worth highlighting that performance in detecting diagnosis-related group differences at this age were significantly better than we reported previously in 5-year-old HIV-infected children where automated segmentation using FreeSurfer version 5.1 detected increases (or decreases) when manual segmentation detected the opposite (Randall et al., 2017).

The findings of this study may be limited to a paediatric sample with prenatal alcohol exposure. However, the relative success of automated FreeSurfer segmentation (especially version 6.0) in this sample of 9-11-year-old children that differs so greatly from the healthy Western adult-based sample used in the development of FreeSurfer (Fischl et al., 2002; Schoemaker et al., 2016), suggests that it can be applied with relative confidence to a geographically different, clinical cohort at this age. Care should be taken in younger populations where differences to an adult-based atlas may be more significant.

Chapter 4: The effects of prenatal alcohol exposure on CC size and inter-hemispheric transfer of tactile information

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Abstract

Prenatal alcohol exposure (PAE) has been associated with structural changes to the corpus callosum (CC) and various behavioural changes, such as compromised inter-hemispheric transfer of tactile stimuli. CC damage may play a role in impaired inter-hemispheric functional connectivity. This study aimed to investigate the effects of PAE and fetal alcohol spectrum disorders (FASD) on inter-hemispheric transfer of tactile information using a finger localization task (FLT) and to investigate potential associations between FLT performance and CC size. Participants were a subset of 16- to 17-year-old children from the Cape Town Longitudinal Cohort, some of whom previously completed MRI at 9-11 years. Alcohol data for this cohort were gathered using the timeline follow-back approach and participants had been diagnosed previously by two expert dysmorphologists as either control, non-syndromal heavily exposed (HE), partial fetal alcohol syndrome (PFAS) or fetal alcohol syndrome (FAS). FLT was administered to 74 participants (32 control, 14 HE, 16 PFAS and 12 FAS) and CC size measured for 34 participants (10 control, 5 HE, 13 PFAS and 7 FAS). Children with FAS showed more transfer-related errors compared to all other groups on the conditions where one finger had been stimulated, either visibly or hidden, effects that remained significant after controlling for potential confounding by IQ and lead. Furthermore, among children with PAE, the number of transfer-related errors on the one-finger/hand hidden condition tended to increase with decreasing CC volume, showing that CC damage in pre-adolescence continues to impact function even into adolescence. This study provides evidence of compromised inter-hemispheric transfer in children with FAS, while those with PFAS or who are non-syndromal appear to be relatively spared.

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4.1 Introduction

Fetal Alcohol Spectrum Disorder (FASD) is the umbrella term used to encompass the range of deficits associated with prenatal alcohol exposure (Hoyme et al., 2005b). Within FASD, fetal alcohol syndrome (FAS) is the most severe diagnosis and is characterized by growth deficits, small head circumference and distinctive craniofacial dysmorphic features. Partial FAS (PFAS) is a less severe diagnosis, characterized by fewer craniofacial dysmorphisms. One of the highest prevalence rates of FAS worldwide is found in the Western Cape Province of South Africa (May et al., 2000; Viljoen et al., 2005). Historic socio-cultural factors have had profound effects on this population, perpetuating the occurrence of prenatal alcohol exposure (May et al., 2013b, 2008). Prenatal alcohol exposure (PAE) results in a diverse range of lifelong behavioural deficits in these individuals (Glass et al., 2014). This has led to studies of structural brain changes in those prenatally exposed to alcohol (see reviews Lebel et al., 2012; Donald et al., 2015) and how these are linked to specific behavioural deficits (Riley et al., 2003).

Previous studies suggest that white matter may be particularly susceptible to the teratogenic effects of prenatal alcohol exposure (PAE) (Archibald et al., 2001; Fan et al., 2016) and many diffusion tensor imaging (DTI) studies have highlighted associations of microstructural changes with cognitive deficits (Ma et al., 2005; Lebel et al., 2008, 2011; Sowell et al., 2008; Fryer et al., 2009; Wozniak et al., 2009, 2011; Fan et al., 2016). The corpus callosum (CC) is one of the most researched white matter structures (Donald et al., 2015b; Lebel et al., 2012; Riley and McGee, 2005) with early autopsy studies reporting abnormal shapes, hypoplasia or, in extreme cases, agenesis (Clarren et al., 1978; Jones and Smith, 1973; Peiffer et al., 1979; Wisniewski et al., 1983).

Neuroimaging of individuals with FASD have demonstrated CC hypoplasia on mid-sagittal MRI scans (Mattson et al., 1992; Sowell et al., 2001; Autti-Rämö et al., 2007; Jacobson et al., 2017), CC displacement (Sowell et al., 2001; Bookstein et al., 2002), and disorganized white matter fibre tracts or myelination disturbances in the CC (Fan et al., 2016; Ma et al., 2005; Sowell et al., 2008; Wozniak et al., 2009). Functionally, thickening of the CC has been linked to deficits in executive function, while a thinner CC has been linked to deficits in motor function (Bookstein et al., 2002) and CC shape abnormalities with verbal memory impairment (Sowell et al., 2001). These findings point to an important role of the CC and

associated WM tracts in behavioural and functional outcomes associated with FASD and PAE.

The CC, which is the largest commissural tract in the brain, plays an important role in transferring information from one side of the cortex to the homologous region of the brain in the opposite hemisphere (Quinn and Geffen, 1986). CC damage may lead to impaired inter-hemispheric functional connectivity, which has been demonstrated in children aged 10-17 years with FASD in medial parietal (para-central) regions connected by posterior callosal fibre projections (Wozniak et al., 2011), and in neonates with PAE between the left and right somatosensory networks (Donald et al., 2016). Inter-hemispheric transfer of tactile information, which relies on an intact CC and somatosensory areas, has been investigated in both normal and clinical populations using the finger localization task (FLT) where participants are asked, after stimulating the tip of a finger (or series of fingers), to report back on which finger(s) were touched (Quinn and Geffen, 1986; Pipe, 1991; Roebuck et al., 2002; Dodge et al., 2009). During crossing conditions, when participants are required to report on the opposite hand from the one touched, the signal is transmitted to the opposite hemisphere via the CC. Specifically, the touch is registered in the ipsilateral primary somatosensory cortex within the parietal lobe from where it is transmitted to the somatosensory association area in the posterior parietal cortex. From here it crosses over via the CC to the contralateral somatosensory association area and is transmitted to the contralateral motor cortex for physical report back by the participant (i.e. indicating on the opposite hand which finger(s) were stimulated) (Quinn and Geffen, 1986). While normal adults performed 7% worse on the FLT when asked to show which finger(s) on one hand were stimulated using their other hand (Geffen et al., 1985), participants who had a partially sectioned midline CC performed 28% worse on crossed conditions. Those with full commissurotomy performed 82% worse. Performance on the FLT is therefore a good proxy for inter-hemispheric transfer of tactile information and could provide insight into the functional integrity of the CC (Geffen et al., 1985).

Roebuck and associates (2002) found that children (aged 8 to 15 years) prenatally exposed to alcohol made more mistakes than the control group when the FLT increased in complexity, and, in a subset for whom neuroimaging data were available, that reduced CC size was related to poorer FLT performance. Our group previously reported impairment in inter-hemispheric transfer of tactile information in pre-adolescent (9-12 years) children with FAS

from Cape Town compared to their highly exposed (including four children with partial FAS) and control counterparts, and in young adults (age 19 years) from the Detroit Longitudinal Cohort in whom transfer-related errors were associated with the quantity of alcohol consumed per occasion by their mothers during pregnancy (Dodge et al., 2009). In a small subset (7 PAE and 7 controls) of children from Cape Town who had been scanned, greater differences between performance on uncrossed and crossed trials were associated with smaller CC size.

In view of our recent finding of decreasing CC size with increasing levels of PAE in 9-11-year-old children from the Cape Town Longitudinal Cohort (Biffen et al., 2018), and our previous finding of poorer performance on the FLT at the same age (Dodge et al., 2009), albeit in a different cohort, we were interested to examine (i) whether impaired inter-hemispheric transfer of tactile information would persist into adolescence and (ii) whether CC size in pre-adolescence would predict FLT performance at this older age. We hypothesized that PAE and smaller CC size would be related to poorer performance on the FLT.

4.2 Methods

4.2.1 Participants

Participants were a subset of 16- to 17-year-old children from the Cape Town Longitudinal Cohort, some of whom previously completed MRI at 9-11 years.

The Cape Town Longitudinal Cohort comprises children born to mothers recruited between 1998-2002 from an antenatal clinic in a Cape Coloured community where prevalence of alcohol use is high (Jacobson et al., 2008). These pregnant women were interviewed at enrolment and during pregnancy using the timeline follow-back approach to obtain a record of their alcohol consumption (Jacobson et al., 2002b). The volume of alcohol consumed was converted to ounces of absolute alcohol (AA), where 1 oz AA is the equivalent of 2 standard drinks. Binge drinking was classified as 5 or more drinks per occasion. Any woman who reported alcohol consumption of at least 1 oz AA/day or at least 2 instances of binge drinking within their first trimester were eligible to participate. Mothers from the same clinic were

invited to participate as controls if they reported minimal alcohol consumption and no binge drinking. The number of cigarettes smoked per day was also recorded.
Exclusion criteria for participation were women younger than 18 years or chronic medical problems, such as diabetes, epilepsy or cardiac problems. Infants from multiple births were excluded, as well as infants presenting with major chromosomal anomalies, seizures and neural tube defects.

The children were examined by two expert dysmorphologists (E.H. Hoyme, M.D., and L.K. Robinson, M.D.) in a clinic held in 2005. Children were examined for growth and FAS-related dysmorphic features using a standard protocol (Hoyme et al., 2005b) based on the Revised Institute of Medicine criteria (Jacobson et al., 2008). The determination of which children met the criteria for diagnosis with FAS or PFAS was made during case conferences with SWJ, JLJ, CDM and the dysmorphologists. If children did not fall into either of these diagnostic groups they were placed, based on maternal alcohol consumption, into either the non-syndromal heavily exposed (HE) group or control group. Five participants could not attend the clinic in 2005 and were seen by another expert dysmorphologist (N. Khaole M.D.). Diagnoses of these children were confirmed by the above dysmorphologists in follow-up clinics in 2009, 2013 and 2016.

4.2.2 Finger Localization Task

In this task, the fingertips of participants were gently touched using a sharpened pencil. Participants were asked to report which finger/s were touched by touching their thumb to the finger stimulated on one hand. The test comprised uncrossed and crossed trials for each of three conditions: (1) one finger touched with hands visible to the participant, (2) one finger touched with hands hidden from the participant, and (3) two fingers touched with hands hidden. In uncrossed trials, the participant touches their thumb to the actual finger stimulated, while for crossed trials they indicate which finger was stimulated on the opposite hand. In the condition where two fingers were touched, the participant was asked to report the stimuli in the same order as they were presented. Each condition was repeated twice – once with fingers on the left hand being stimulated, and once with those on the right hand being stimulated.
A cardboard box with windows on both sides (one covered with a cloth for the participants to insert their hands into and one for the researcher to administer the test through) was used for the hidden conditions. Before each condition the participant was told whether one or two

fingers would be touched and whether they were expected to report back on the same or opposite hand. Instructions where administered in the participant's home language (English or Afrikaans) to make the task as clear as possible. Self-corrections were allowed. No feedback regarding performance was given by the examiner. The researcher was blind to participant diagnosis, except in extreme cases where FAS was clearly apparent.

For each condition and each hand, the number of errors on uncrossed and crossed trials were recorded separately, and the difference between the number of errors on crossed and uncrossed trials (CUD) computed. Since CUD's represent the increase in transfer-related errors compared to other errors, this was used as a proxy for inter-hemispheric transfer deficiency.

4.2.3 Segmentation Protocol

In a subset of the children for whom FLT data were collected, the CC had previously been manually traced by a single, blinded neuroanatomist on MRI scans acquired at ages 9-11 years (Biffen et al., 2018). Briefly, CC area was obtained by averaging Mulitracer "frust" volumes (Woods, 2003) of CC's traced on two contiguous midline sagittal slices. CC volumes were obtained from the same image set by performing automated segmentation with FreeSurfer v 6.0 (http://surfer.nmr.mgh.harvard.edu/).

4.2.4 Statistical Analyses

All statistical analyses were performed using SPSS version 25 (SPSS Inc, Chicago, IL). Two factor repeated measures analysis of variance (ANOVA) was used to examine main effects of inter-hemispheric transfer and stimulated hand, as well as potential interaction effects of stimulated hand on inter-hemispheric transfer. Crossed/uncrossed differences, as a proxy for inter-hemispheric transfer deficits, on each FLT condition were compared between diagnostic groups using ANOVA, and associations with extent of prenatal alcohol exposure were examined using Spearman correlation.

Two maternal (smoking and education) and 5 child characteristics (age, sex, total intracranial volume (TIV), lead exposure, and IQ) were considered as potential confounders. ANCOVA and multiple linear regression were used to control on subsequent analyses for any control variable found to be weakly associated ($p \leq 0.1$) with CUD's on any FLT condition.

Within the subset of children for whom structural MRI data were available, we examined associations of crossed/uncrossed differences with CC areas and volumes, respectively, using Pearson correlation. Multiple linear regression was used to control for potential confounding.

4.3 Results

Sample characteristics are summarized in Table 4.1. Groups did not differ by sex or age at FLT. Children with FAS or PFAS had lower IQs than those in the HE and control groups, and their mothers completed fewer years of formal education. All alcohol exposed children had higher levels of lead exposure than non-exposed controls, and their mothers smoked more cigarettes and consumed higher levels of alcohol during pregnancy than mothers of children in the control group. Only one mother in the control group reported drinking during pregnancy and she only consumed 2 drinks per occasion on 2-3 days during pregnancy.

Table 4.1: Sample characteristics (N=74)

		Control (*n*=32)		Exposed HE (*n*=14)		PFAS (*n*=16)		FAS (*n*=12)		TOTAL (*N*=74)		*F* or χ^2
Child:												
Sex: Male	*n*(%)	19	(59%)	8	(57%)	10	(63%)	6	(50%)	43	(58%)	1.946
Age at FLT (yr)	Mean(SD)	16.1	(0.8)	16.4	(0.7)	16.7	(0.7)	16.7	(0.7)	16.4	(0.8)	2.616†
WISC-IV IQ[a]	Mean(SD)	77	(12)	80	(11)	64	(10)	65	(10)	73	(13)	8.066***
Lead (ug/dl)[b]	Mean(SD)	6.75	(2.94)	10.29	(3.24)	10.75	(6.34)	11.75	(4.67)	9.10	(4.64)	6.061***
Maternal:												
Cigarettes/day[c]	Median(IQR)	0.0	(4.0)	6.3	(11.4)	7.0	(6.3)	6.0	(9.5)	3.0	(7.5)	6.723***
Mother's education[d]	Mean(SD)	10.0	(1.8)	9.1	(2.5)	6.9	(2.4)	8.3	(2.2)	8.9	(2.4)	7.822***
Alcohol across pregnancy:												
AA/day (oz)[e]	Median(IQR)	0.00	(0.00)	1.04	(0.80)	1.02	(0.61)	0.81	(1.11)	0.42	(1.01)	35.840***
AA/drinking day (oz)[f]	Mean(SD)	0.04	(0.21)	4.79	(3.50)	3.60	(1.24)	4.51	(1.70)	2.43	(2.75)	36.270***
Proportional drinking days/wk[g]	Mean(SD)	0.00	(0.00)	0.27	(0.14)	0.31	(0.14)	0.26	(0.24)	0.16	(0.19)	29.768***

For skewed data medians and interquartile ranges (IQR) were used. HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS; WISC-IV Wechsler Intelligence Scales for Children-Fourth Edition.

[a] FAS < HE and Control (*p*'s≤0.003); PFAS < HE and Control (*p*'s≤0.001)
[b] Control < FAS, PFAS and HE (*p*'s≤0.01)
[c] Control < FAS, PFAS and HE (*p*'s≤0.009)
[d] Control > FAS and PFAS (*p*'s≤0.018); HE > PFAS (*p*=0.006)
[e] Control < FAS, PFAS, HE (*p*'s<0.001)
[f] Control < FAS, PFAS, HE (*p*'s<0.001)
[g] Control < FAS, PFAS, HE (*p*'s<0.001)
†$p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Performance on the FLT for the group as a whole is summarised in Table 4.2. A significant main effect of transfer was detected in all 3 conditions; in all conditions more errors were made when information had to cross the CC (Fig. 4.1). No effects of hand or transfer by hand interactions were detected. Due to the absence of effects of hand, the number of errors on left and right trials of each condition were summed for subsequent analyses. Figure 4.1 shows the number of errors by diagnostic group separately for uncrossed and crossed trials of each condition. While performance on uncrossed trials was similar across groups on the two one-finger conditions, children with FAS made more errors than control children on the uncrossed trials of the most difficult two-finger/hand hidden condition. On crossed trials, children with FAS performed worse than other groups in all three conditions.

Table 4.2: Repeated measures ANOVA to examine main and interaction effects of inter-hemispheric transfer (crossed vs uncrossed) and stimulated hand (left vs right) (N=74).

	Inter-hemispheric Transfer			Stimulated Hand			Transfer x Hand Interaction
	Uncrossed	**Crossed**	***F* (*p*)**	**Left**	**Right**	***F* (*p*)**	***F* (p)**
One-finger /hand visible	0.05 (0.17)	0.27 (0.59)	**10.108 (0.002)**	0.16 (0.33)	0.16 (0.43)	0.016 (0.900)	0.858 (0.357)
One-finger /hand hidden	0.10 (0.32)	0.74 (1.07)	**36.936 (<0.0001)**	0.36 (0.59)	0.48 (0.88)	1.706 (0.196)	0.395 (0.532)
Two-finger /hand hidden	2.15 (2.13)	4.08 (2.75)	**64.726 (<0.0001)**	3.21 (2.44)	3.02 (2.37)	0.822 (0.368)	0.024 (0.877)

Values are mean (SD)

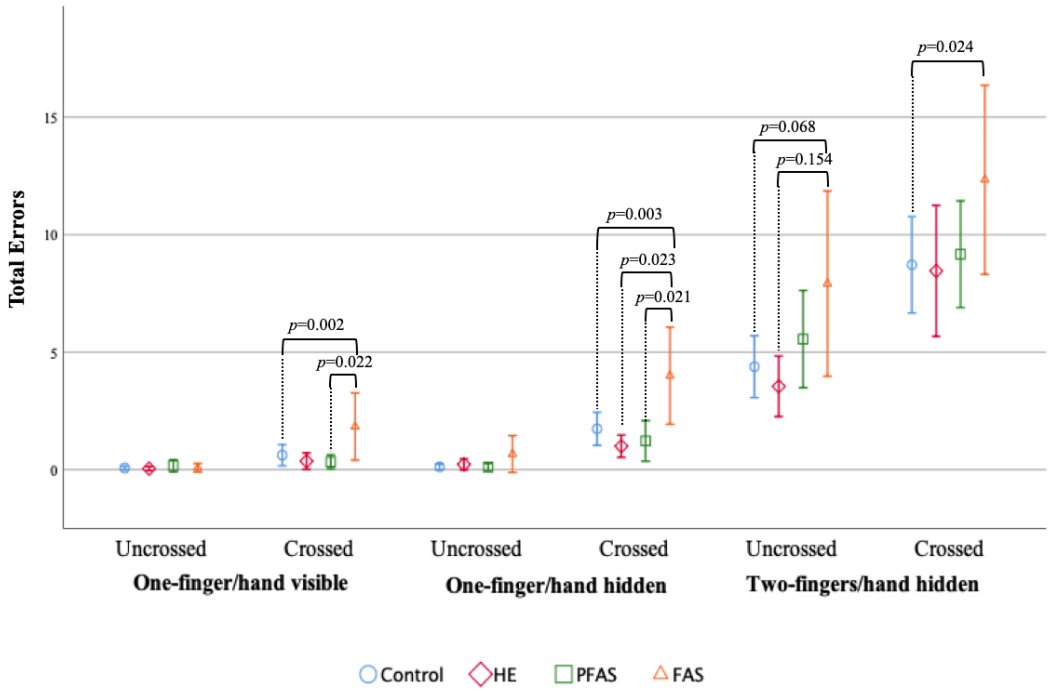

Figure 4.1: Number of errors (mean ± 95% confidence intervals) by diagnostic group on uncrossed and crossed trials of each condition. Significant and trending Kruskal-Wallis pairwise comparisons are indicated.

Of the potential confounders considered, increasing lead exposure and decreasing IQ were both associated with increasing crossed/uncrossed differences on both the one-finger/hand hidden and two-finger/hand hidden conditions (Table 4.3).

Table 4.3: Association of total number of crossed/uncrossed differences (CUD) on each FLT condition with potential confounders (N=74).

FLT Condition	Age at FLT	Sex	Lead (ug/dl)	IQ	Mother's education	Cigarettes per day	TIV[#]
One-finger/ hand visible	0.093 (0.431)	-0.055 (0.641)	0.079 (0.505)	-0.018 (0.876)	-0.101 (0.392)	-0.091 (0.440)	-0.178 (0.307)
One-finger/ hand hidden	0.084 (0.477)	0.076 (0.519)	**0.241 (0.038)**	**-0.354 (0.002)**	0.061 (0.609)	0.090 (0.443)	-0.032 (0.857)
Two-finger/ hand hidden	0.178 (0.128)	-0.059 (0.619)	**0.204 (0.081)**	**-0.303 (0.009)**	-0.147 (0.210)	-0.002 (0.986)	-0.055 (0.754)

Values are *r*(*p*); *r* is Spearman *rho*; Bold print denotes significance at $p<0.10$

[#]TIV only available for 35 participants who were scanned.

On both the one-finger conditions (hand visible and hand hidden), children with FAS made significantly more transfer-related errors than children in all other groups (Table 4.4 and Figure 4.2). These differences survived after controlling for potential confounding by lead and IQ. In contrast, no associations of CUD's with extent of prenatal alcohol exposure were found on any of the FLT conditions (all *p*'s > 0.10; Table 4.5).

Table 4.4: Comparison between diagnostic groups on total number of crossed/uncrossed differences for each FLT condition (N=74)

	Control (*n*=32)	**Exposed** (*n*=42)			***F*** **(*p*)**	***F*[1]** **(*p*)**	***F*[2]** **(*p*)**
		HE (*n*=14)	**PFAS** (*n*=16)	**FAS** (*n*=12)			
One-finger/ hand visible[#]	0.19 (0.64)	0.21 (0.70)	0.19 (0.75)	1.75 (2.22)	**6.940 (<0.001)**	**6.804 (<0.001)**	**6.613 (0.001)**
One-finger/ hand hidden[#]	0.88 (1.26)	0.64 (1.22)	1.13 (1.82)	3.33 (2.35)	**8.064 (<0.001)**	**5.963 (0.001)**	**5.944 (0.001)**
Two-finger/ hand hidden	3.84 (4.14)	3.36 (3.97)	3.88 (5.43)	4.42 (4.08)	0.136 (0.938)	0.217 (0.884)	0.322 (0.809)

Values are mean (SD). HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS

Bold print denotes significance at $p\leq0.05$

[1]Controlling for IQ

[2]Controlling for IQ and lead

[#] FAS>PFAS,HE and control groups (all *p*'s≤0.009).

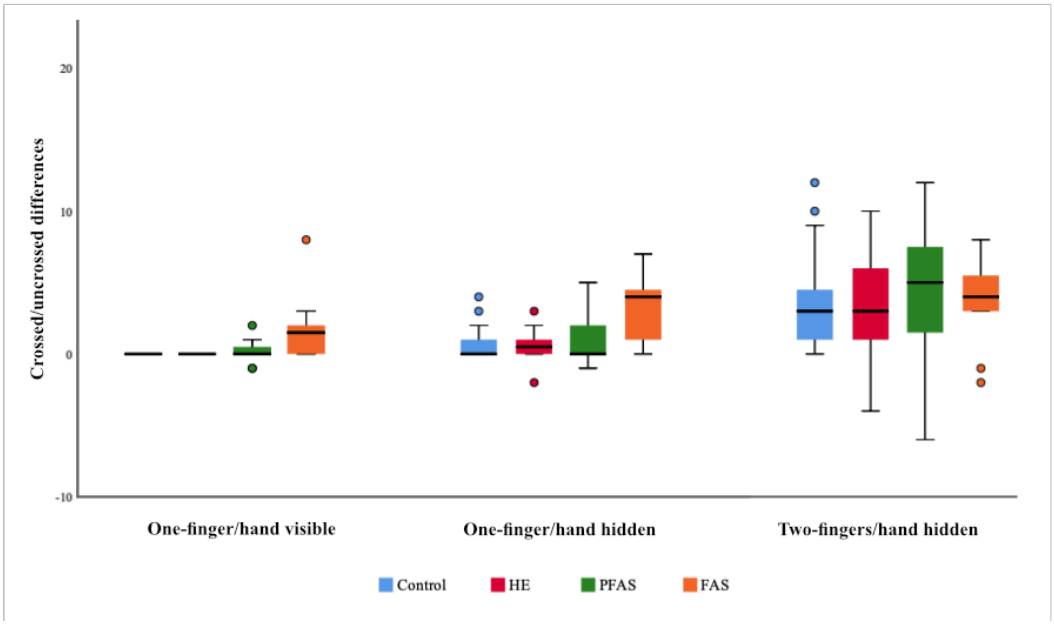

Figure 4.2: Box-and whisker plots of crossed/uncrossed differences (CUD) for each condition by diagnostic group.

Table 4.5: Association of total number of crossed/uncrossed differences on each of the FLT conditions with level of prenatal alcohol exposure (N=74).

	AA/day	**AA/drinking day**	**Drinking days**
FLT Condition	*r* *(p)*	*r* *(p)*	*r* *(p)*
One-finger/hand visible	0.048 (0.683)	0.142 (0.226)	0.037 (0.755)
One-finger/hand hidden	0.105 (0.371)	0.190 (0.104)	0.008 (0.496)
Two-finger/hand hidden	0.093 (0.430)	0.057 (0.631)	0.129 (0.272)

r Spearman's *rho*

Table 4.6: Sample characteristics for sub-sample that were scanned (N=35).

		Control (*n*=10)		Exposed HE (*n*=5)		PFAS (*n*=13)		FAS (*n*=7)		TOTAL (*N*=35)		*F* or χ^2
Child:												
Sex: Male	*n*(%)	6	(60%)	4	(80%)	8	(62%)	3	(43%)	21	(60%)	1.400
Age at scan (yr)[a]	Mean(SD)	10.6	(0.5)	11.4	(0.7)	10.5	(0.5)	9.5	(0.9)	9.77	(0.9)	4.048**
Age at FLT (yr)	Mean(SD)	16.7	(0.3)	17.0	(0.3)	16.9	(0.6)	16.9	(0.6)	16.8	(0.5)	0.363
WISC-IV IQ[b]	Mean(SD)	73	(6)	80	(13)	66	(10)	69	(11)	70	(11)	6.630**
TIV ($x10^6$ mm^3)[c]	Mean (SD)	1.40	(0.11)	1.46	(0.08)	1.39	(0.14)	1.23	(0.14)	1.38	(0.14)	8.064***
Lead (ug/dl)	Mean(SD)	8.30	(2.31)	10.80	(0.84)	10.77	(6.92)	11.14	(4.88)	10.14	(4.90)	1.495
Maternal:												
Cigarettes/day[d]	Median(IQR)	0.0	(3.6)	5.5	(8.3)	8.8	(6.4)	8.7	(6.1)	6.1	(5.9)	2.442†
Mother's education[e]	Mean(SD)	10.0	(2.3)	9.8	(2.3)	7.7	(2.7)	9.7	(2.1)	8.7	(2.5)	10.347***
Alcohol across pregnancy:												
AA/day (oz)[f]	Median(IQR)	0.00	(0.00)	0.73	(0.66)	1.04	(0.84)	0.81	(2.49)	0.7	(1.07)	20.463***
AA/drinking day (oz)[g]	Mean(SD)	0.12	(0.37)	3.23	(1.38)	4.27	(1.56)	4.73	(1.96)	2.82	(2.19)	19.946***
Proportional drinking days/wk[h]	Mean(SD)	0.00	(0.00)	0.20	(0.09)	0.33	(0.14)	0.31	(0.30)	0.21	(0.21)	16.648***

For skewed data medians and interquartile ranges (IQR) were used. HE heavily exposed non-syndromal; FAS fetal alcohol syndrome; PFAS partial FAS; WISC-IV Wechsler Intelligence Scales for Children-Fourth Edition; TIV total intracranial volume as measured by FreeSurfer v. 6.0.

[a] HE > FAS (p=0.038), PFAS (p=0.001), Control (p=0.042)

[b] FAS < HE (p=0.006), Control (p=0.023); PFAS < HE (p<0.001), Control (p=0.003)

[c] FAS < PFAS (p=0.003), HE (p<0.001), Control (p≤0.001)

[d] FAS > Control (p=0.028); PFAS > Control (p=0.025)

[e] PFAS < FAS (p=0.003), HE (p<0.001), Control (p<0.001)

[f] Control < FAS, PFAS, HE (all p's<0.001); HE < FAS, PFAS (both p's≤0.001)

[g] Control < FAS, PFAS, HE (all p's<0.001)

[h] Control < FAS, PFAS, HE (all p's≤0.001), HE < FAS, PFAS (both p's≤0.005)

†p≤0.1, * p≤0.05, **p≤0.01, ***p≤0.001

Table 4.6 presents the sample characteristics for the subset of children for whom imaging data were available. Demographics were similar to that of the whole sample, except that in this sub-sample, lead exposure did not differ between diagnostic groups. Children in the HE diagnostic group were slightly older at the time of scanning than those in the other groups, and those with FAS had smaller TIV's compared to children in all other groups.

Within the sample as a whole and those prenatally exposed to alcohol, we did not find any associations on any of the FLT conditions between the number of transfer-related errors and CC area from manual tracing (Table 4.7A). However, among children with PAE, the number of transfer-related errors on the one-finger/hand hidden condition tended to increase with decreasing CC volume, a finding that survived after control for differences in age at

scanning, IQ and lead exposure. This trend was not evident in the control children (Supplementary Table). The absence of this association in the control group suggests that CC size impacts inter-hemispheric transfer of tactile information more in children with PAE than in controls.

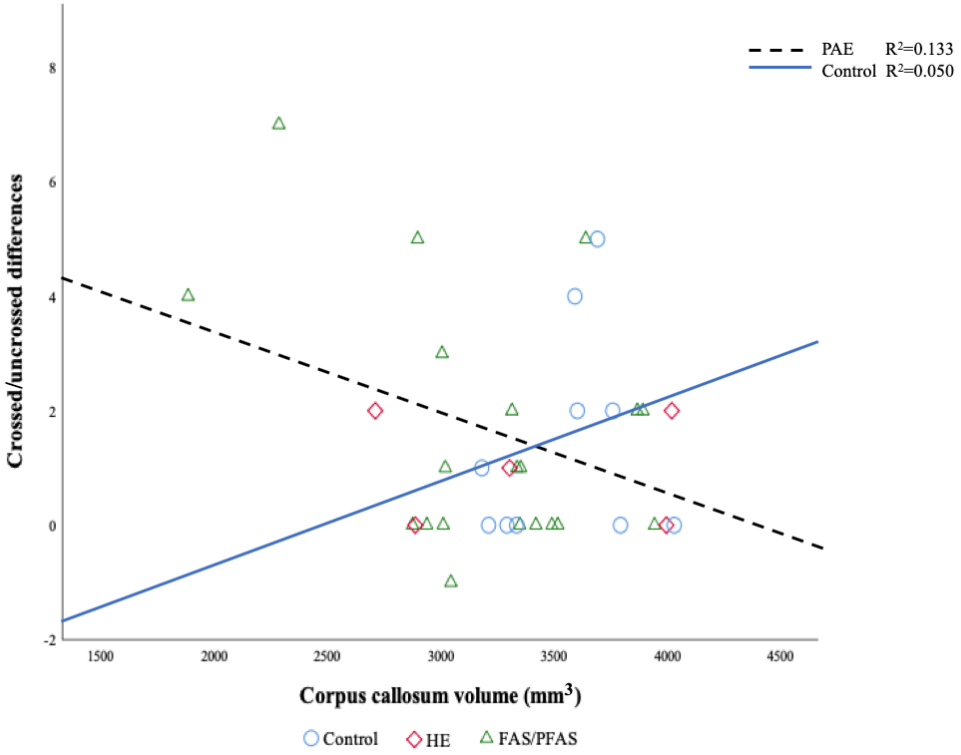

Figure 4.3: Scatter-plots showing crossed/uncrossed differences on the one-finger/hand hidden condition as a function of corpus callosum volume (mm3). The black dashed line shows the relationship in children with prenatal alcohol exposure (PAE), and the solid blue line that in control children.

Table 4.7: Associations in the sample as a whole, and within the PAE group separately, of crossed/uncrossed differences (CUD) for each FLT condition with (A) areas and (B) volumes of the corpus callosum (CC) at age 9-11 years.

Condition	All Children (N=35)					PAE (n=25)				
	r (*p*)	β^1 (*p*)	β^2 (*p*)	β^3 (*p*)	β^{all} (*p*)	*r* (*p*)	β^1 (*p*)	β^2 (*p*)	β^3 (*p*)	β^{all} (*p*)
A) Associations with CC areas (mm^2) from manual tracing										
One-finger/ hand visible	-0.047 (0.791)	-0.198 (0.278)	-0.137 (0.462)	-0.223 (0.237)	-0.220 (0.252)	-0.017 (0.937)	-0.357 (0.162)	-0.162 (0.472)	-0.369 (0.115)	-0.357 (0.206)
One-finger/ hand hidden	-0.103 (0.555)	-0.055 (0.773)	-0.044 (0.813)	-0.027 (0.892)	-0.030 (0.882)	-0.220 (0.291)	-0.218 (0.421)	-0.182 (0.432)	-0.210 (0.449)	-0.258 (0.392)
Two-finger/ hand hidden	0.008 (0.964)	-0.005 (0.979)	0.096 (0.608)	0.057 (0.770)	0.058 (0.770)	0.024 (0.908)	0.227 (0.398)	0.150 (0.513)	0.243 (0.372)	0.361 (0.212)
B) Associations with CC volumes (mm^3) from automated segmentation										
One-finger/ hand visible	-0.129 (0.460)	-0.259 (0.142)	-0.162 (0.356)	-0.258 (0.149)	-0.260 (0.164)	-0.159 (0.973)	-0.352 (0.105)	-0.211 (0.308)	-0.343 (0.124)	-0.334 (0.166)
One-finger/ hand hidden	-0.260 (0.131)	-0.238 (0.196)	-0.239 (0.171)	-0.240 (0.196)	-0.263 (0.174)	**-0.365 (0.073)**	-0.370 (0.103)	**-0.347 (0.097)**	**-0.388 (0.095)**	**-0.454 (0.070)**
Two-finger/ hand hidden	0.037 (0.831)	0.031 (0.871)	0.069 (0.693)	0.027 (0.884)	0.031 (0.873)	0.011 (0.960)	0.104 (0.654)	0.051 (0.808)	0.085 (0.718)	0.167 (0.507)

Bold print denotes significance at $p \leq 0.10$

r Pearson correlation coefficient

β^1 after control for age at scan (yrs)

β^2 after control for IQ

β^3 after control for age at scan (yrs) and IQ

β^{all} after control for age at scan (yrs), IQ and lead

Supplementary Table: Associations in the control children and those with FAS/PFAS of crossed/uncrossed differences (CUD) for each FLT condition with (A) areas and (B) volumes of the corpus callosum (CC) at age 9-11 years.

	Controls (n=10)					**FAS/PFAS** (n=20)				
FLT Condition	*r* (*p*)	β^1 (*p*)	β^2 (*p*)	β^3 (*p*)	β^{all} (*p*)	*r* (*p*)	β^1 (*p*)	β^2 (*p*)	β^3 (*p*)	β^{all} (*p*)
(A) Associations with CC areas (mm^2) from manual tracing										
One-finger/ hand visible CUD	-0.171 (0.636)	-0.051 (0.907)	-0.177 (0.642)	-0.073 (0.876)	0.035 (0.942)	0.106 (0.665)	-0.282 (0.222)	0.001 (0.996)	-0.315 (0.211)	-0.304 (0.247)
One-finger/ hand hidden CUD	0.345 (0.328)	0.353 (0.416)	0.338 (0.351)	0.320 (0.479)	0.358 (0.486)	-0.226 (0.338)	-0.308 (0.281)	-0.257 (0.348)	-0.321 (0.302)	-0.332 (0.308)
Two-finger/ hand hidden CUD	-0.023 (0.950)	0.249 (0.538)	-0.028 (0.941)	0.232 (0.594)	0.263 (0.594)	0.068 (0.777)	0.140 (0.628)	0.178 (0.513)	0.218 (0.482)	0.270 (0.392)
(B) Associations with CC volumes (mm^3) from automated segmentation										
One-finger/ hand visible CUD	0.012 (0.973)	0.020 (0.959)	-0.186 (0.699)	-0.148 (0.773)	-0.042 (0.938)	-0.089 (0.710)	**-0.380 (0.067)**	-0.157 (0.523)	**-0.385 (0.074)**	**-0.379 (0.092)**
One-finger/ hand hidden CUD	0.224 (0.535)	0.219 (0.566)	0.076 (0.873)	0.043 (0.933)	0.061 0.915	**-0.405 (0.077)**	**-0.476 (0.061)**	**-0.419 (0.085)**	**-0.476 (0.072)**	**-0.490 (0.076)**
Two-finger/ hand hidden CUD	0.196 (0.587)	0.208 (0.553)	0.103 (0.831)	0.173 (0.719)	0.199 (0.760)	0.091 (0.704)	0.137 (0.607)	0.140 (0.576)	0.158 (0.565)	0.201 (0.469)

[#]One FAS/PFAS child did not complete any of the hidden FLT tests

Bold print denotes significance at $p \leq 0.10$

r Pearson correlation coefficient

β^1 after control for age at scan (yr)

β^2 after control for IQ

β^3 after control for age at scan (yr) and IQ

β^{all} after control for age at scan (yrs), IQ and lead

4.4 Discussion

The aims of this study were to examine whether alcohol-related impairment in inter-hemispheric transfer of tactile information observed at younger ages persist into adolescence and whether CC size in pre-adolescence predicts FLT performance at this older age. Although we did not find any significant associations between level of alcohol exposure and transfer-related errors, children with FAS made more transfer-related errors compared to all other groups when one finger was stimulated – either visibly or hidden. These findings suggest that the effect of alcohol exposure on inter-hemispheric transfer is not dose dependent, but rather a threshold effect with only the most severely affected children being deficient in this domain. There were no differences between groups on the number of transfer-related errors in the more complex two-finger/hand hidden condition, presumably due to the greater number of errors in all groups on the uncrossed trials in this condition, but especially among children with FAS, thus reducing the CUD's in this group. Among children prenatally exposed to alcohol, there was a negative relation between CUD scores on the one-finger/hand hidden condition and CC volume that fell short of statistical significance.

Our finding here of a threshold effect is consistent with that reported previously on the one-finger/hand hidden condition in a cross-sectional cohort of children aged 8-12 years from Cape Town where a deficit in inter-hemispheric transfer was found only in children with FAS (Dodge, et al. 2009), but not in a heavily exposed group that also included 4 children with PFAS. In contrast, Roebuck and associates (2002) found greater crossed/uncrossed differences, indicative of inefficient inter-hemispheric transfer of information, across one- and two-finger hand hidden trials (but not in the one-finger/hand visible condition) in 8-15-year old children with PAE compared to control children. Since 64% of the children in their PAE group had been diagnosed with FAS, it is, however, possible that their finding was largely attributable to those children. The threshold effect could not be evaluated in the young adults from the Longitudinal Detroit cohort, as only 2 of the 63 alcohol exposed participants in that study had been diagnosed with FAS (Dodge et al., 2009).

In contrast to Dodge et al. (2009) who found increasing transfer-related errors on both the one- and two-finger hand hidden conditions with more concentrated drinking per occasion by mothers of the young adults from the Detroit Longitudinal cohort, we observed only a weak relationship that fell short of statistical significance ($r = 0.190$, $p = 0.104$) between drinks per

occasion and CUD's on the one-finger/hand hidden condition in the adolescents studied here. No other associations with continuous measures of alcohol exposure were observed. Notably, Dodge et al. (2009) did not report any associations of continuous measures of alcohol exposure with FLT performance in the 8-12-year old children from the cross-sectional cohort in Cape Town in whom information regarding alcohol exposure collected retrospectively may not be as accurate. Taken together, findings from the two longitudinal cohorts in whom inter-hemispheric transfer of information has been studied suggest that more concentrated drinking may be more detrimental.

Similar to findings from Dodge et al. (2009) and Roebuck et al. (2002), participants across all groups made more errors on the most complex condition where two fingers were stimulated compared to conditions where only one finger was stimulated. While all groups performed similarly on uncrossed trials of conditions where one finger was stimulated, children with FAS made more errors than control children on *both* uncrossed and crossed trials of this more challenging condition. Roebuck et al. (2002) also reported more errors in children with PAE than controls on the two-finger trials. The fact that children with FAS made more errors on this condition not only on the crossed trials involving inter-hemispheric transfer, but also on the uncrossed trials, resulting in similar crossed/uncrossed differences across groups, suggests that poorer performance on this condition is related to lower IQ rather than deficits in inter-hemispheric transfer. Notably, group differences in CUD's on the two one-finger conditions remained significant after control for IQ and lead, confirming that these are not due to the lower IQ of children with FAS.

Although we did not find associations of CC volumes at pre-adolescence with performance at this later age on the visible one-finger condition, there was a trend-level association among alcohol exposed children (but not controls) of decreasing CC volumes with increasing transfer-related errors on the one-finger/hand hidden condition – effects that got stronger after excluding the 5 non-syndromal HE children (Supplementary Table). Dodge et al. (2009) similarly found negative associations of CUD's with areas of the isthmus and splenium on the same condition in a cross-sectional cohort of younger children from Cape Town. In contrast to the two Cape Town studies, Roebuck et al. (2002) did not find association of CUD's on the one-finger/hand hidden condition with total CC area, but instead found that smaller CC areas were associated with increasing CUD's on the more complex two-finger condition, effects that got stronger after excluding the 6 control children (37.5%) in their

sample. It has been shown previously that damage to the CC results in poorer performance as inter-hemispheric task complexity increases (Banich and Shenker, 1994), possibly due to more brain regions becoming involved and needing to communicate effectively. Failure in our sample to detect a relationship between CC volumes and performance on the more complex condition, supports our earlier premise that performance on the two-finger condition is impacted more by IQ than inefficient inter-hemispheric transfer.

Anatomically, the corpus callosum undergoes the biggest growth before age 10, but continues its maturation until adulthood (Quinn and Geffen, 1986) and normally developing children have been shown to perform better on the more complex FLT conditions with increasing age (Pipe, 1991; Quinn and Geffen, 1986). Improved performance with age on the more complex condition is seen also when comparing the errors reported in the three FLT studies examining effects of PAE, with the younger children from the Cape Town cross-sectional cohort (Dodge et al., 2009) and the Roebuck et al. (2002) study making more errors on the two-finger trials than the young adults from the Detroit Longitudinal cohort (Dodge et al., 2009) and adolescents studied here. The fact that we do not find associations of CC volumes with FLT performance in controls, suggests that their CC's may have matured significantly over the 6 years since the MRI scan such that their CC size at that earlier age is irrelevant. In contrast, CC damage in alcohol exposed children appear to persist and still impact performance on inter-hemispheric transfer 6-7 years later. These findings suggest that CC damage related to PAE may be permanent rather than developmental delay, which should be further investigated in future studies involving longitudinal brain imaging data.

It was unexpected in our study to find associations of inter-hemispheric transfer performance with CC volumes from automated FreeSurfer segmentation, but *not* with areas from "gold-standard" manual tracing. This suggests that area on a midline sagittal slice may not be an appropriate proxy for CC size, as the volume across which it extends in adjoining slices may significantly impact function. Notably, Dodge, et al. (2009) made use of the Brain 2 automated segmentation programme, while Roebuck, et al. (2002) employed a semi-automated tracing protocol (Riley et al., 1995). Briefly, the outer borders of the CC were manually traced on a mid-sagittal CC slice, after which a programme was used to automatically divide it into 5 areas.

Directionality of inter-hemispheric transfer was also investigated. Similar to the findings of Roebuck et al. (2002), we did not find any effect of hand or transfer by hand interaction, confirming that the results of the FLT are bidirectional and not influenced by which hand was stimulated. In contrast, Dodge, et al. (2009) did find effects between hand and condition, although the direction showing greater impairment differed for different conditions and in the different cohorts they studied.

The limitations of this study include that the MRI scans obtained for these participants were obtained at a mean age of 10.7 (±0.6) years while the FLT was performed at the mean age of 16.9 (±0.4) years. Although age-matched MRIs would have been preferred, this design allowed us to make some inferences as to the extent to which early CC damage persists into adolescence and affects future function. Notably, associations of CC volume with FLT performance survived after control for group differences in age at scanning. Although MRI data were only available for a sub-sample of the children studied, the sub-sample size was still more than double that of the sub-samples for whom MRI data were available in the Roebuck et al. (2002) and Dodge et al. (2009) studies.

This study supports and expands upon previous findings that PAE causes subtle differences in inter-hemispheric transfer of tactile information. Although our findings in adolescence support previous evidence in a pre-adolescent Cape Town cohort (Dodge et al., 2009) that alcohol exposure related deficits in inter-hemispheric transfer are diagnostic rather than dose dependent, affecting only children diagnosed with FAS, binge drinking does appear to be more detrimental. Furthermore, among adolescents with PAE, reduced CC size in pre-adolescence is related to worse inter-hemispheric transfer.

Chapter 5: Discussion and conclusion

5.1 Discussion

The results of the first study, using manual tracing, found that increased total consumption and frequency of PAE were associated with volume reductions bilaterally in the caudate nuclei and hippocampi and a smaller CC area, with the latter partially mediating effects of PAE on IQ. Within the FAS/PFAS diagnostic group, poorer CVLT-C scores were associated with smaller CC area and larger hippocampal volumes. Notably, regional reductions in size were all disproportionate to overall total brain volume reduction. Furthermore, the effects of continuous measures of PAE were more significant than diagnostic groups in this sample, and the only structure that showed differences by diagnostic group was the right hippocampus. Comparison of volumes obtained from automated analyses using FreeSurfer versions 5.1 and 6.0 to those from manual segmentation, supported findings from other studies that agreement and consistency of methods differ depending on the complexity and contrast of the structure under consideration. Within the subcortical structures, larger structure size and increased contrast, for example, assisted in segmentation of the caudate nuclei where greater agreement was seen than in the hippocampi. Both agreement and consistency were poor in the CC, presumably due to differing segmentation protocols between automated and manual methods. Unexpectedly, automated segmentation identified more regions with alcohol-related volume reductions than manual tracing, although these "potentially spurious" findings did not survive after controlling for reductions in TIV. The third study demonstrated that alcohol-related impairment in inter-hemispheric transfer of tactile information observed previously at younger and older ages, is seen in children with FAS during adolescence, and is related among children with PAE to reductions in CC size (albeit at a younger age). These findings suggest that alcohol-related effects on CC size impact function even in adolescence and potentially throughout life, while inter-hemispheric transfer abilities in non-exposed controls appear to be independent of early CC size. Notably, inter-hemispheric transfer deficits were not significantly associated with levels of alcohol exposure, but rather appeared to be a diagnostic effect.

Many studies have investigated the effect of PAE on volumes of various structures with a focus on either binary measures of PAE or between diagnostic group differences (see reviews Lebel et al., 2011; Donald et al., 2015). The advantage of using the time-line follow-back

approach (Jacobson et al., 2008) in conjunction with diagnostic groups is that both categorical and continuous measures of PAE could be investigated in the studies of this dissertation, which may provide a more complete picture of the deleterious effects of PAE on the investigated structures and behavioural outcomes. The strength of using prospective data in this thesis is its accuracy as compared to retrospectively ascertained maternal alcohol data (Jacobson et al., 2002, 2008), which could be collected years after the pregnancy. There is an argument that neuroanatomical and neurobehavioural deficits may be semi-independent of craniofacial dysmorphology (Bookstein et al., 2002; Connor et al., 2006; Wozniak et al., 2011), despite its key role in diagnosing individuals with FAS (Hoyme et al., 2005, 2016). This is because craniofacial dysmorphology seems to be linked to a very specific time-frame of PAE - the gastrulation stage (3-4 weeks) of embryological development (Guerri et al., 2009; Sulik, 2005). Whereas the effects of PAE on neural development have been shown to occur within this stage as well as weeks 7-20, when the brain undergoes another critical stage of development (Guerri et al., 2009; Miller, 2007, 1995; Nixon and Crews, 2002). This is where the increased accuracy of prospectively collected continuous alcohol data is most informative in understanding the effects of PAE on the developing brain.

No between diagnostic group differences were found in regional volumes in the caudate nuclei, NA, left hippocampus and CC using the manual tracing protocol, with trending differences in the right hippocampus. When compared to the automated tracing protocol, the older version of FreeSurfer (v. 5.1) found group related differences in the right caudate nucleus, bilateral NA and hippocampi and CC, but these seem to have been resolved to more closely match manual tracing by v. 6.0, which showed no group differences after controlling for TIV. Yet, continuous measures of PAE (AA/day and drinking days per week) were associated with all structures, except the NA, in both manual tracing and both versions of FreeSurfer. After accounting for TIV these structures remained significant across methods, except for the CC, which remained as a trend. This is comparable to various other studies investigating the effects of PAE in participants from Cape Town, which also found continuous measures of PAE to show increased sensitivity, as compared to diagnostic groups, on brain structure and function (De Guio et al., 2014; Meintjes et al., 2014; du Plessis et al., 2015). Conversely, inter-hemispheric transfer deficits were seen in only the FAS diagnostic group and did not show significant associations with continuous measures of PAE. One of the limitations of human studies as compared to animal studies is that we cannot control when PAE occurs, yet understanding if an effect is dose dependent or a threshold effect that only

occurs within diagnostic groups can help with our understanding of the teratogenic mechanisms of PAE on brain structure and function and in informing studies focusing on timed interventions.

ROIs examined in this dissertation were investigated, in part, due to their association with some of the neurobehavioural deficits reported in children who have been prenatally exposed to alcohol. The caudate nucleus is involved in everyday executive functions, like cognitive flexibility, planning, response inhibition and concept formation and reasoning (Mattson et al., 1996, 2001; Fryer et al., 2007a). Decreased volume within the left and right caudate nuclei with PAE in this sample supports existing literature reporting similar findings (Mattson et al., 1994, 1996, 1999, 2001; Archibald et al., 2001; Roussotte et al., 2012). Being a larger subcortical structure with very clear borders with delineation that is well agreed upon in the literature (Archibald et al., 2001; Fryer et al., 2012; Looi et al., 2008), it was expected that the agreement and consistency between manual and automated segmentation methods would be highest in this region. The reliability between manual tracing and even the older version of FreeSurfer, which was less similar to manual tracing than FreeSurfer v 6.0, was good in this region. This finding supports previous studies that also found the caudate nucleus to show the best agreement between manual and automated tracing (Akudjedu et al., 2018; Dewey et al., 2010). It also supports that findings within the literature are more consistent and informative when the tracing methods employed have higher agreement.

The nucleus accumbens was considered mainly due to the protocol used in manual tracing of the caudate nucleus. As previously discussed, it can only be truly differentiated from the caudate nuclei histologically, but it has to be removed in order to investigate the volume of the caudate nuclei independently. This is done with a straight line, which is not representative of the organic shapes of these subcortical structures. The caudate is a larger structure and is more robust to these slight inorganic variations, but the NA seems to be greatly affected. This can perhaps be seen best in the lack of consistency and agreement between manual and automated segmentation within this structure, even between FreeSurfer versions. Despite our findings agreeing with those of Archibald and associates (2001), who also failed to find an association between PAE and NA volume, these findings might be due to either tracing methods or a relative sparing of the NA in these individuals. With further advances in MRI, such as higher resolution structural imaging, volumetric measurement by both manual tracing and automated segmentation may become a more valid and reliable measure of the effects of

PAE on the NA. As it stands, the current consistent findings lead us to conclude that some midline brain structures, such as the caudate nucleus, may be more susceptible than others to PAE during development (Guerri et al., 2009; Meintjes et al., 2014; Sulik et al., 1981; Zhou et al., 2003) and that the NA seems relatively spared.

The right hippocampus was the only structure to show a trend-level decrease in volume between FAS/PFAS and control groups. While no significant between-group differences were observed between hippocampal size and diagnostic criteria in previous studies, the left hippocampus was observed to be smaller in children with PAE, but they did not test structure size on a diagnostic level, which may account for these differences in findings (Autti-Rämö et al., 2007; Willoughby et al., 2008). The size of the hippocampi were affected bilaterally over a continuous range of alcohol consumption, whether or not prenatal alcohol consumption results in a FASD diagnosis. The left and right hippocampus were found to decrease in volume as the volume of absolute alcohol consumed increased. These differences remained after accounting for intracranial volume, which is a neurobiological signature of FASD (Clarren and Smith, 1978; Archibald et al., 2001; Hoyme et al., 2005; Lebel et al., 2011; Donald et al., 2015) The hippocampi also decreased with an increase in frequency of alcohol consumption, with the left hippocampus remaining significant after controlling for confounders. Although our findings for the caudate nuclei, nucleus accumbens and CC agree with those of Archibald and associates (2001), who used a similar tracing protocol as this dissertation, their results differed in that they did not find significant differences in hippocampal volumes. The clinical implications of volume changes in the hippocampus, discussed in Chapter 3, has made it region of interest for comparison of manual and automated tracing programmes (Akudjedu et al., 2018; Barnes et al., 2008; Dewey et al., 2010; Morey et al., 2009; Schoemaker et al., 2016). There was an increase in correlation, consistency and absolute agreement with manual tracing from FreeSurfer v. 5.1 to v. 6.0. The decreased reliability between manual and automated segmentation protocols in the hippocampus compared to the caudate nucleus may be due to the smaller structure volume and differences in tracing, such as, whether or not they include the alveus in the hippocampal volume. All these considerations may also contribute to differences in findings in the literature on the effects of PAE on the hippocampus.

Often studies that investigate neuroanatomical changes related to PAE do not relate structure to function (Archibald et al., 2001; Astley et al., 2009). Functional deficits can often be

inferred from previous studies, but this assumes similarities between diverse populations and tracing protocols, which can be problematic. Many studies have demonstrated the role of the hippocampus in memory acquisition and consolidation (see review by Gluck et al., 2007), thus, the effect of PAE on the relationship between hippocampal volume and verbal memory performance may be more evident. In one study of adults with PAE and dysmorphic features, greater volume of the right hippocampus was associated with more efficient encoding and better recall of verbal information (Coles et al., 2011). In children with FASD, hippocampal volume has been found to have a positive association with verbal recall scores (Willoughby et al., 2008). This may help in understanding the finding of smaller left and right hippocampi in PAE children with increased exposure to alcohol measures, but, within the FAS/PFAS group, larger hippocampi related to poorer scores on the CVLT-C total learning scores. This is possibly due to poorer neural pruning within this group, resulting in less efficient neural pathways leading to poorer overall cognitive function in this domain, but, without supporting histological data this is difficult to interpret. Another consideration is that the hippocampus may have domain specific functions, such as encoding, which also influences pruning patterns. Previous neurobehavioural tests on this sample reported impaired encoding (i.e. information acquisition) but spared retention, which was in agreement with the theory that the negative effect of alcohol on verbal learning is due to deficits in encoding the information, rather than impaired recall or retrieval (Lewis et al., 2015).

Structurally and functionally, the CC has been shown to be particularly vulnerable to the effects of PAE (e.g. Mattson et al., 1992; Sowell et al., 2001, 2008; Bookstein et al., 2002; Ma et al., 2005; Autti-Rämö et al., 2007; Fryer et al., 2009). The results of this dissertation further support and add to these findings. PAE was associated with smaller CC after controlling for lead and TIV in Chapter 2. CC volume has been shown to be highly influenced by maternal smoking during pregnancy (Jacobsen et al., 2007; Jacobson et al., 2008; Paus et al., 2008), so it was not surprising that the effects of alcohol and smoking were confounded in this sample. Further investigation suggested that in a larger sample size their effects would likely be independent.

An aim of this dissertation was to relate structural changes to neurobehavioural deficits in children with PAE. Chapter 2 found larger CC area was associated with higher IQ scores. Reductions in CC are were shown to partially mediate the effect of PAE on IQ and emphasizes the role that structure can play on function. Another study in the same cohort,

reported that microstructural changes within two areas of the CC mediate the effects of PAE on IQ (Fan et al., 2016). The underlying premise is that the CC facilitates information transfer from one hemisphere to the other and that damage resulting from PAE to this system may contribute to the functional deficits seen in the literature (Quinn and Geffen, 1986). Although the exact nature of the damage to the CC may range from myelination deficits to decreased axonal number, these result in a measurable structural change (Bookstein et al., 2002; Lebel et al., 2008; Sowell et al., 2008). The problem with tracing the CC is that there is little consensus on exact tracing protocol. This results in CC measurements that cannot easily be compared across manual and automated segmentation, nor within the existing literature. However, the literature on the effects of PAE on CC does seem to converge to some extent. For example, despite using different techniques to measure the CC it seems that decreased CC size in children with PAE is related to decreased performance on the FLT, especially with increased complexity (Roebuck et al., 2002; Dodge et al., 2009). Using FreeSurfer v. 6.0, which was shown to be more similar to manual tracing than v 5.1, we also found that decreased CC volume in the PAE group was associated with increased errors when one finger was stimulated and the participant's hand hidden. This seems to support that smaller CC volume results in poorer inter-hemispheric transfer in PAE individuals. Yet, this was not seen when examining associations of manual tracing to FLT performance. When Chapter 3 compared the manual and automated methods of tracing, however, it showed that for the CC manual and automated methods were vastly different and very difficult to compare directly. The main difference being that our manual tracing protocol examines the average area of the CC in two midline sagittal slices, while automated segmentation with FreeSurfer calculates a volume for the CC over multiple slices. Although this may provide a more complete picture of the morphology of the CC as a 3-dimentional structure, which may be more closely linked to how it functions to transfer tactile information between hemispheres, it is difficult to know where one would end the tracing if a similar protocol was followed for manual tracing. This further emphasises that methods of measuring structures can result in different findings and must be interpreted with care. Given that FreeSurfer v. 6.0 provided comparable results to manual tracing on structures that are considered "difficult" to trace, like the hippocampus, instils confidence, even when results differ from manual tracing. Appreciation of the differences in protocols are essential, however, to be able to appropriately consider the impact of these differences on relationships with behavioural outcomes.

One of the limitations of this dissertation is that the regions investigated are responsible for diverse functional outcomes. As such, a larger battery of neuropsychological tests could have provided more comprehensive insights into structure-function relationships and potential effects of PAE on these relationships. While the children studied here have been assessed on a broad range of neuropsychological tests as part of ongoing studies of FASD being performed in Cape Town, inclusion of these data was outside the scope of the current project. Further, the longitudinal nature of this ongoing research is that in some cases timelines of tests performed is not ideal. In the case of Chapter 4, for example, the MRI scans used to measure CC area and volume were from scans obtained at 9-11 years old, while the FLT was performed at 16-17 yrs. This was due to MRI scanning of these participants at adolescence not being performed yet. However, this limitation highlighted that early neurological damage in the CC only within the PAE children had effects on future functioning and shows the long-term behavioural implications of PAE. Future work would benefit from analysing MRI data collected from these participants during adolescence and comparing it to the FLT data collected for this thesis. Comparing children to adolescents also raises the question of whether additional behavioural questions need to included. In particular, alcohol consumption and drug use by adolescents should be considered as these may have deleterious effects on the brain concomitant with or in addition to the teratogenic effects of prenatal alcohol exposure. Another limitation is that manual tracing was only compared to FreeSurfer versions 5.1 and 6.0. Despite this programme being popular in the literature, it is not the only automated segmentation programme that is used. Future studies might consider other automated segmentation programmes, especially as updates occur, as this dissertation found that the latest version of FreeSurfer had vastly improved in terms of inter-rater reliability to manual tracing, providing more comparable clinical findings. Finally, the fact that CC segmentation protocols differed between manual and automated methods meant that the manual and automated results could not be directly compared. This ROI was of particular interest to this and many other studies in the literature, given its increased vulnerability to the teratogenic effects of PAE. Future work could greatly benefit from the development of a single tracing protocol for this area for all morphometric studies.

5.2 Conclusion

The findings of this dissertation confirm previous literature, finding disproportionate decreases in subcortical volumes of the caudate nuclei and hippocampi with increasing levels of PAE. PAE was also associated with disproportionate reductions in the CC that partially mediated the effects of PAE on IQ, and was related within children with PAE with poorer inter-hemispheric transfer of tactile information. Investigation into how different tracing methods can influence structural and clinical literature associated with PAE showed that, overall, automated segmentation identified more structures to be affected by PAE. However, controlling for TIV removed many of these and produced results that were more similar to manual tracing when using the latest version of FreeSurfer. This highlights that findings from studies with different tracing methods should be interpreted and compared with due caution. Overall, this body of work adds to the growing body of literature showing subcortical GM changes with PAE and re-affirms that WM is particularly susceptible to structural and functional deficits associated with PAE. The functional consequences of PAE on the CC include decreased IQ, poorer performance on the CVLT-C, and inter-hemispheric transfer deficits in children with FAS. These findings emphasize the deleterious consequences of PAE on the developing fetus.

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